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# Polyethenoid Fatty Acid Metabolism. II. Deposition of Polyunsaturated Fatty Acids in Fat-Deficient Rats Upon Single Fatty Acid Supplementation <sup>1</sup>

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## INTRODUCTION

Linoleic acid (1) and arachidonic acid (2) have been shown to prevent the symptoms characterized by poor growth and skin lesions developed by rats on a fat-deficient diet. Supplementation of other unsaturated fatty acids such as linolenic acid (3) and docosahexaenoic acid (3,4) were shown to increase growth but to have little effect in preventing or curing the skin symptoms. Nunn and Smedley-Maclean (5) reported evidence indicating the synthesis of more highly unsaturated fatty acids in the rat from dietary linoleic acid and linolenic acid.

It has been demonstrated that fatty acids containing four and more double bonds exist in animal tissues (5,6). Riekehoff *et al.* (7) found that polyunsaturated fatty acid content of tissue lipides was greatly influenced by fat deficiency and dietary fat. They also found that deposition of polyethenoid fatty acids was most pronounced in the hearts of rats held on a fat-deficient diet prior to supplementation. Liver, brain, kidney, and muscle also contained relatively large amounts of these fatty acids, whereas skin and depot fat contained only traces.

<sup>1</sup> Abstracted from a thesis presented by Carl Widmer, Jr. to the Graduate Faculty of Agricultural and Mechanical College of Texas in partial fulfillment of the requirements for the degree of Master of Science, June 1949. A preliminary report was made at the meeting of the American Chemical Society, San Francisco, March 1949 (Abstracts p. 5c). Supported in part by grants from the Office of Naval Research and the National Dairy Council on behalf of the American Dairy Association.

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There was strong evidence to show that the rat probably synthesizes arachidonic acid from linoleic acid.

The purpose of this work was to study the effects of stearate, olcate, linoleate, and linolenate in the diet upon the synthesis and deposition of polyunsaturated fatty acids in fat-deficient rats. Previous work does not prove conclusively that linoleic acid is a metabolic precursor of arachidonic acid, nor is knowledge available concerning linolenic acid metabolism. This investigation was initiated in the hope that such information could be gained.

### EXPERIMENTAL

Weanling rats were placed on a fat-deficient diet for 3 months before the beginning of the experiment. The diet consisted of purified casein, sucrose, salts, cystine, and vitamin E containing the water-soluble vitamins essential for the rat. Water was provided *ad lib*. Supplements of vitamins A and D were administered in adequate amounts in the form of a concentrate.

The animals were divided into 6 groups consisting of 12-15 animals each. The initial control group was sacrificed at the end of the 3-month depletion period. The terminal control group was killed at the same time the experimental groups were killed. Daily supplements of 0.05 ml./animal of ethyl stearate, ethyl oleate, ethyl linoleate, and ethyl linolenate were given to the four remaining groups for a period of 8 weeks. In addition a seventh group was included in the experiment, consisting of rats fed a normal stock diet, so that comparison could be made with animals whose diet was essentially complete. This group is designated as the normal control group.

Animals were sacrificed by decapitation and the blood was collected. Each group was then divided into subgroups 1 and 2 as homogeneously as possible, dissected, and the organs pooled. The dissected animals contributed to 10 pools of organs: heart, liver, kidney, brain, blood, muscle, intestine, skin, adipose tissue, and carcass.

The minced tissue was extracted twice by refluxing in an Erlenmeyer flask with a 3:1 mixture of alcohol-ether. The dehydrated tissue was then extracted with ether. When the extract solution was concentrated to a convenient volume, the fat was saponified with KOH, cooled, and the unsaponifiables were extracted with ether. This was followed by acidification and extraction of the free fatty acids with petroleum ether. The petroleum ether was evaporated under vacuum and the fatty acids were sealed in vacuum ampoules until they could be analyzed.

The spectrophotometric determination of the polyunsaturated fatty acids was made using the alkali isomerization technique developed by Holman and Burr for analysis of the polyunsaturated fatty acids (8). Briefly, this consisted of heating a sample of the mixed fatty acids in 5.0 ml. of 23.0% KOH-ethylene glycol reagent at 180°C. for 8.0 min. The reaction mixture was then diluted to 100.0 ml. Spectral density readings were taken at the appropriate wavelengths with the spectrophotometer, making additional dilutions when necessary. The extinction coefficients were corrected for "background" absorption. Average  $E_{1\text{cm}}^{1\%}$  values were calculated for the organ lipides of each group of experimental animals at the absorption peak wavelength characteristic of each of the three polyenoic acids.

## RESULTS

If the extinction coefficients of pure standards are known, it is possible to calculate the percentages of various polyunsaturated fatty acids in the fatty acid mixture. Although pure standards of all the polyenoic acids are not available, certain approximations can be made. A sample of methyl docosahexaenoate generously supplied by Dr. D. A. Sutton was available for use as a provisional standard for hexaenoic acids. This preparation was impure (I. V.:371, theoretical, 446) but was employed in order that approximations might be made. The  $E_{1\text{cm}}^{1\%}$  at 3750 Å (characteristic of conjugated hexaene) developed upon alkaline isomerization of this preparation was found to be 157. Pentaenoic acid standard was not available, nor have extinction coefficients been determined as yet for this substance after isomerization. The extinction coefficient for the characteristic absorption bands developed from linoleate, linolenate, arachidonate, and docosahexaenoate were found to be close to a straight line when plotted against wavelength. By interpolation the value for pentaenoic acid extinction coefficient was estimated to be  $E_{1\text{cm}}^{1\%} = 300$ . Using these values, and assuming that the pentaene contribution to light absorption at 3000 Å is in the same order as that of the hexaene standard, tentative calculations of 4, 5, and 6 double-bond acid contents could be made.

Extinction coefficients for the various samples of tissue fatty acids are presented in Figs. 1, 2, and 3, and the approximate percentages of the polyunsaturated fatty acids in rat tissues lipides are given in Table I. It is to be emphasized that the latter values are only tentative and will be subject to revision when pure standards are available.

*Heart*

See Fig. 1. Normal heart fatty acids are estimated to contain about 12% hexaenoic acid, 0% pentaenoic acid, and 7% tetraenoic acid. The deficient controls and those animals fed stearate, oleate, and linoleate showed hexaenoic acid concentration in the heart fatty acids to be less than 7% and pentaenoic acid to be absent. In these same groups, with the exception of the linoleate group, tetraenoic acid concentration was found to be less than 3.5%. The linoleate group, on the other hand, showed a tetraenoic acid concentration in the heart fatty acids of 9.5%, more than a twofold increase, indicating the biological synthesis of arachidonic acid from linoleic acid as was suggested by previous

TABLE I  
*Estimated Polyunsaturated Fatty Acid Content of Rat Tissue Fatty Acids*

	Tetraene	Pentaene	Hexaene
Heart			
	%	%	%
Normal	7.3	0.0	12.2
Initial	3.2	0.0	2.7
Terminal	3.2	0.0	5.9
Stearate	3.5	0.0	4.8
Oleate	2.4	0.0	6.9
Linoleate	9.5	0.0	4.7
Linolenate	4.7	0.0	14.0
Liver			
Normal	9.8	0.0	10.4
Initial	1.4	0.0	5.0
Terminal	0.2	0.0	4.5
Stearate	0.9	0.0	4.0
Oleate	0.8	0.0	5.5
Linoleate	6.6	0.0	3.5
Linolenate	0.3	0.0	11.6
Muscle			
Normal	1.9	0.0	3.3
Initial	0.7	0.0	1.3
Terminal	0.3	0.0	1.8
Stearate	0.3	0.0	1.5
Oleate	0.5	0.0	2.0
Linoleate	1.1	0.0	1.6
Linolenate	0.7	0.0	3.1
Carcass			
Normal	1.8	0.0	2.3
Initial	1.0	0.0	1.7
Terminal	0.5	0.0	1.5
Stearate	0.8	0.0	1.4
Oleate	0.9	0.0	2.0
Linoleate	1.3	0.0	1.3
Linolenate	1.3	0.0	3.4

TABLE I—*Continued*

	Tetraene	Pentaene	Hexaene
Intestine			
	%	%	%
Normal	1.8	0.0	1.9
Initial	0.8	0.0	1.7
Terminal	1.1	0.0	2.0
Stearate	0.4	0.0	2.1
Oleate	0.2	0.0	1.6
Linoleate	0.9	0.0	1.4
Linolenate	0.8	0.0	2.0
Adipose Tissue			
Normal	0.1	0.0	1.7
Initial	0.0	0.0	0.8
Terminal	0.0	0.0	1.5
Stearate	0.0	0.0	1.6
Oleate	0.0	0.0	1.3
Linoleate	0.0	0.0	1.9
Linolenate	0.0	0.0	0.9
Skin			
Normal	0.6	0.0	1.5
Initial	0.0	0.0	1.5
Terminal	0.0	0.0	1.1
Stearate	0.0	0.0	1.7
Oleate	0.0	0.0	1.6
Linoleate	0.3	0.0	1.3
Linolenate	0.0	0.0	1.1
Kidney			
Normal	4.2	0.0	1.3
Initial	1.9	0.0	1.1
Terminal	1.7	0.0	0.9
Stearate	2.1	0.0	1.0
Oleate	1.5	0.0	1.0
Linoleate	4.3	0.0	0.9
Linolenate	2.4	6.3	4.0

TABLE I—*Continued*

	Tetraene	Pentaene	Hexaene
Blood			
	%	%	%
Normal	3.2	0.0	3.1
Initial	3.8	0.0	0.7
Stearate	2.7	0.0	0.0
Oleate	1.9	0.0	1.5
Linoleate	4.9	0.8	1.1
Linolenate	1.6	8.3	11.0
Brain			
Normal	2.8	0.0	6.9
Initial	1.6	0.0	5.0
Terminal	2.3	0.0	4.7
Oleate	2.0	0.0	8.3
Linoleate	4.5	0.0	6.0
Linolenate	0.6	0.0	11.3

workers. The linolenate group showed 14% hexaenoic acid deposited in the heart fatty acids, or twice that found in the deficient control group. In this sample no pentaenoic acid was found, but tetraenoic acid was present in amount comparable to that found in the deficient control groups. This suggests the synthesis of six-double-bond acid from three-double-bond acid. The change in the polyunsaturated acids of the tissues in response to dietary changes are more pronounced in the heart than in any other of the tissues studied.

### *Liver*

See Fig. 1. Polyunsaturates were also concentrated in high amounts in the liver fatty acids. Normal liver fatty acids were found to contain over 10% hexaenoic acid, 0% pentaenoic acid, and 9.8% tetraenoic acid. The stearate, oleate, linoleate, and deficient control groups all contained less than 5.5% hexaenoic acid, no pentaenoic acid, and excepting the linoleate group about 1.0% tetraenoic acid. The linoleate group contained 6.6% tetraenoic acid in the liver lipide, a fourfold increase, again indicating the synthesis of arachidonic acid from linoleic acid. Similarly,

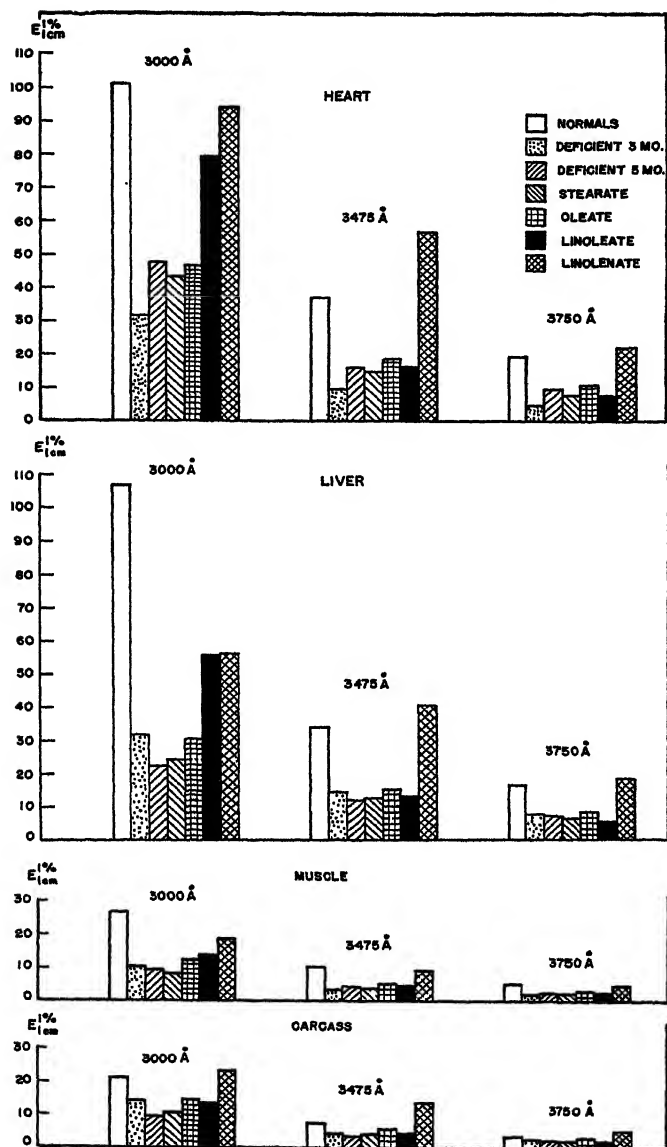


FIG. 1. Extinction coefficients for isomerized tissue fatty acids from heart, liver, skeletal muscle, and carcass of rats on different fatty acid supplements. 3750 Å = 6 double bonds; 3475 Å = 5 double bonds; 3000 Å = 4 double bonds.

the linolenate group showed synthesis of hexaenoic acid from trienoic acid with 11.6% hexaenoic acid, 0% pentaenoic acid, and 0.3% tetraenoic acid in the fatty acids.

### *Skeletal Muscle and Carcass*

See Fig. 1. Fatty acids extracted from skeletal muscle and carcass have approximately the same relative composition as heart and liver, but with much smaller quantities of the polyunsaturates.

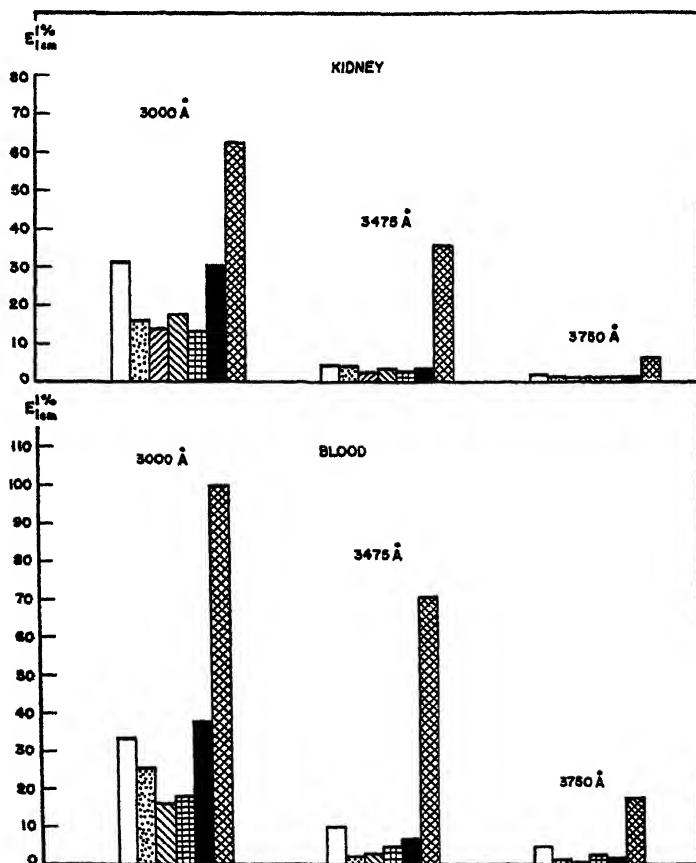


FIG. 2. Extinction coefficients for isomerized fatty acids of blood and kidney. See legends for Fig. 1.

*Blood and Kidney*

See Fig. 2. These tissues are discussed together since they have similar fatty acid composition with respect to the polyunsaturated acids. The kidney lipide of all groups, except the linolenate group, exhibit very low percentages of hexaenoic acid. Kidney fatty acids from the latter group contain 4% hexaenoic acid. However, an indication of the synthesis of five-double-bond fatty acid appears here for the first time. The linolenate kidney fatty acids are estimated to contain 6% pentaenoic acid, whereas, kidney fatty acids from other groups contain no pentaenoic acid. The deficient controls and the stearate and oleate groups exhibit the same trends in kidney lipide composition as they do in other tissue lipides already mentioned. Again the linoleate group shows the synthesis of arachidonic acid, its kidney fatty acid content close to that of the normal control group. The linolenate group shows a low concentration of tetraenoic acid in the kidney fatty acids, a value of roughly 2.4%, as compared to less than 2% found in the kidney lipide of the deficient controls. As was stated before, the blood shows a similar picture, only the effect of linolenate in the diet is more pronounced. A rather interesting phenomenon is presented by the observation that only blood and kidney fatty acids seem to contain pentaenoic acid as the result of linolenate supplementation, its absence being indicated in the other tissues examined. Kidney damage is one of the chief results of essential fatty acid deficiency. The observed appearance of pentaenoic acid in kidney and blood after supplementation with linolenate may be the result of impaired function of kidney tissue and may possibly be an abnormal synthesis.

*Adipose Tissue, Skin and Intestine*

See Fig. 3. Due to the low concentration of polyunsaturates in these tissues, the effects of dietary supplementation are hardly noticeable.

*Brain*

See Fig. 3. Brain lipide was found to contain rather large quantities of polyunsaturated fatty acids, but dietary supplementation had a lesser effect upon its composition, the changes being similar to other tissues.

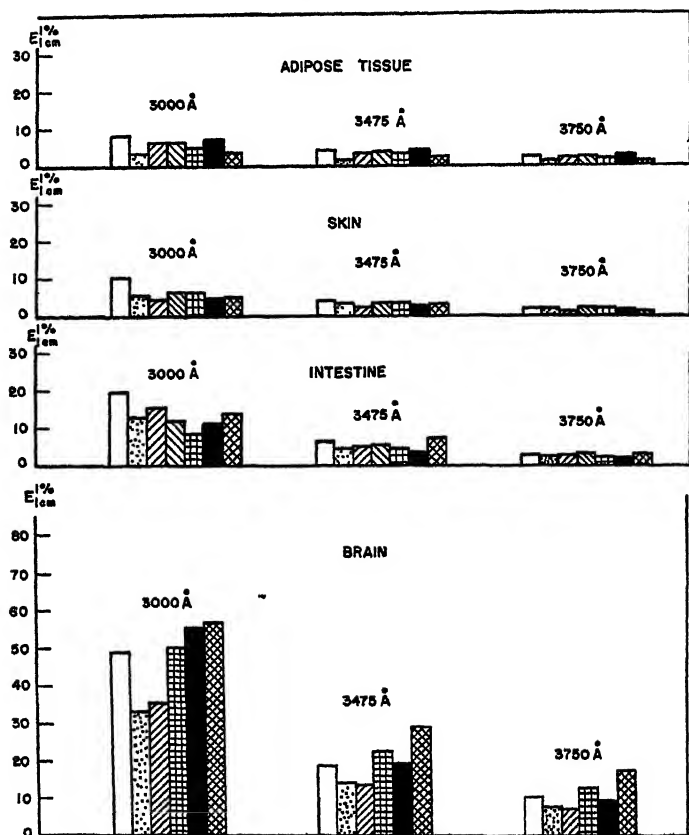


FIG. 3. Extinction coefficients for isomerized fatty acids of adipose tissue, skin, intestine, and brain. See legends for Fig. 1.

### DISCUSSION

The data shown here confirm the report of Riekehoff *et al.* (7) that the polyunsaturated acids present in tissue fatty acids decrease markedly when the animal is reared on a fat-deficient diet. Within three months on such a diet, the tetraenoic and hexaenoic acids had decreased markedly, the change being greatest in the heart fatty acids. It should be mentioned also that Riekehoff *et al.* demonstrated that this decrease in polyunsaturated acids was accompanied by a striking increase in trienoic acid, a type of acid normally present in low quantities, if at all. This change was also found greatest in the heart.

A comparison of polyunsaturated acid contents of the various muscles is of interest. The intestine, consisting largely of smooth muscle, contained small quantities of polyunsaturates in its fatty acids, and differences in dietary fatty acids caused only slight variations in composition. This was likewise true for skeletal muscle. However, cardiac muscle fatty acids were found to contain large amounts of polyunsaturates and these varied strikingly with dietary fatty acid. These differences in muscle fatty acid composition are highly interesting and should merit further study.

The data show quite clearly that deposition of tetraenoic acid does not occur when linolenate is fed, nor does hexaenoic acid when linoleate is fed. The groups fed olcate and stearate showed no noticeable response with respect to their polyunsaturated fatty acid composition and remained quite like the deficient controls.

Hexaenoic acid is normally present in rat tissues in higher concentrations than previously supposed. However it is questionable whether it is as important a constituent of tissues as is arachidonic acid, since arachidonic acid produces good growth response and cures skin disorders, whereas, hexaenoic acid promotes weight increase only.

Fat deficiency reduces the amount of hexaenoic acid in heart and liver to about the same extent, but the retention of tetraene by the heart is considerably greater than by the liver. Kidney and blood lie between liver and heart in this respect. The polyunsaturated fatty acids of the heart seem to be more responsive to changes in the fatty acid of the diet than do those of the other organs.

It is clear that a 3-month depletion period is sufficient to change the polyunsaturated fatty acid composition of rat tissues to a very noticeable extent. Similarly, it is clear that a daily dose of 30 mg. of essential fatty acids for 8 weeks, a total of 1.8 g., is sufficient to cause a marked response in the deposition of polyunsaturated acid.

It is also concluded that deposition of six-double-bond acid represents a different dietary source from deposition of four-double-bond acid. That is, deposition of hexaenoic acid, probably occurs only when a plant oil containing linolenic acid or an animal oil containing hexaenoic acid is present in the diet. Likewise, it is probable that arachidonic acid is deposited only when an animal fat is fed, or when a vegetable oil containing linoleic acid is present in the diet. Arachidonic and hexaenoic acids probably also serve different functions in the tissue metabolism as judged from their differing curative effects upon fat deficiency. Thus "fat deficiency" is probably a double deficiency which can be complete-

ly relieved only by administration of linoleic acid, or arachidonic acid and linolenic acid, or hexaenoic acid.

### SUMMARY

1. Supplements of 0.05 ml. of ethyl stearate, oleate, linoleate, and linolenate per day were fed to each of four groups of fat-starved rats for a period of 8 weeks. Spectrophotometric analysis of the alkali-isomerized samples of fatty acids extracted from the various tissues was made.

2. Rats fed a normal stock ration showed deposition of polyunsaturated fatty acids in the heart, liver, brain, kidney, blood, and skeletal muscle, arranged in order of decreasing concentration. Negligible amounts were found to be present in adipose tissue.

3. The rat was shown to synthesize arachidonic acid from linoleic acid but not from linolenic acid.

4. The rat was shown to synthesize hexaenoic acid from linolenic acid but not from linoleic acid. Thus, tetraenoic and hexaenoic acids in tissues arise from two dietary sources.

5. Synthesis of some pentaenoic acid from linolenic acid was also shown. It occurs mainly in kidney and blood.

6. Tetraene and hexaene are normally found in tissue fatty acids of the rat on a stock diet. The apparent synthesis of pentaenoic acid from linolenic acid by deficient animals probably represents an abnormality since its absence is consistently demonstrated in all other experimental groups.

7. Very noticeable changes in the polyunsaturated fatty acid composition of rat tissues are evident after three months depletion period.

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# Enzymic Synthesis of Peptide Bonds. I. Some Factors Which Influence the Synthesis of Peptide Bonds as Catalyzed by Papain <sup>1</sup>

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## INTRODUCTION

This paper presents some details of a systematic investigation (1) of factors controlling the enzyme-catalyzed synthesis of peptide bonds as exemplified by the formation of acylamino acid anilides, with papain as the catalyst (2). Such data are of interest with respect to an intrinsic understanding of normal and abnormal peptide bond synthesis in biological systems as well as to an understanding of the control of peptide bond synthesis by inhibitors (3). Systematic study of such systems may also lead to an increasingly factual basis for evaluation of the validity of the device of synthesis of insoluble anilides as a biological model. These studies, in conjunction with information obtained in other ways (4), have in fact made it possible to assess the reasons for quantitative differences in anilide syntheses.

During this study, the importance of buffer concentration has been particularly evident. In earlier work, the usual concentrations of citrate buffer have been 0.2 *M* or less (2,5). When more concentrated citrate buffer such as 1.0 *M* is employed, the reaction frequently proceeds more rapidly and in better over all yield under standard conditions such as a 3-day time (Fig. 1). This not only gives more of the *L* form but facilitates preparation of the *D* form in resolutions since the latter is less

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contaminated by the acyl-L-amino acid. The data presented in Fig. 1 for benzoylglycinanilide are typical of many of the results obtained. In one synthesis of benzoyl-L-alaninanilide the conditions were such that 1.0 *M* buffer caused greater than a 20-fold difference in yield at the optimum pH.

This type of behavior may be explainable as an instance of general acid catalysis, in which the buffer-substrate interaction is a rate-limiting step. Because of the occurrence of buffers in various biological systems, such a means of regulation of enzyme activity may not be without biological significance.

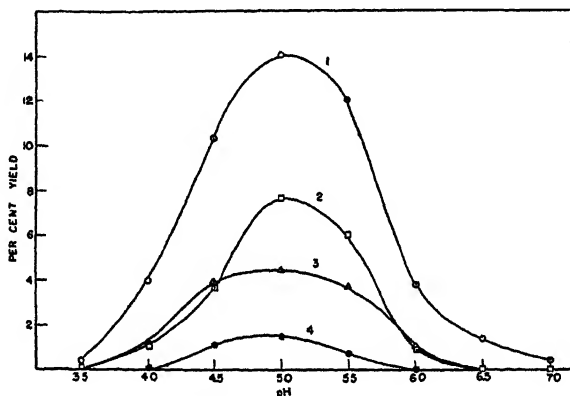


FIG. 1. Effects of pH, benzoylglycine concentration, and citrate buffer concentration on yield of benzoylglycinanilide. Curve 1. 1.0 *M* buffer, 0.005 moles of benzoylglycine. Curve 2. 1.0 *M* buffer, 0.0025 moles of benzoylglycine. Curve 3. 0.1 *M* buffer, 0.005 moles of benzoylglycine. Curve 4. 0.1 *M* buffer, 0.0025 moles of benzoylglycine.

A quantitative evaluation of the effect of concentration of all the reactants is brought out in Fig. 2. With the amounts of all components held constant, the total volume of buffer was varied for both benzoylglycine and benzoyl-DL-alanine. These results are of course in agreement with kinetic considerations of concentration, but present definite values.

In Table I are presented the effects of variations of a number of conditions in the synthesis of benzoyl-L-alaninanilide. The effects of alteration of any one condition seem in much of this work to depend upon the combination of other conditions employed. In the experiment reported, controlled variations revealed that a 6-day incubation gave

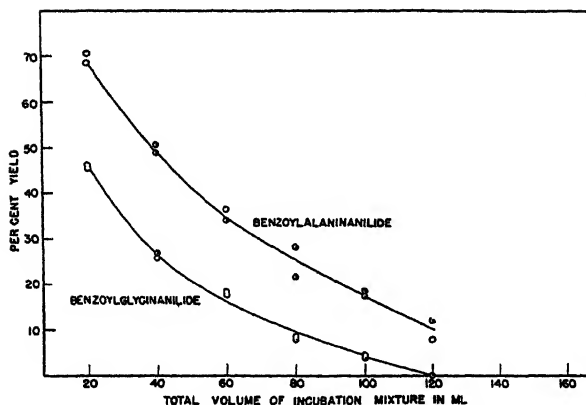


FIG. 2. Effect of total volume of incubation mixture on yields of two acylamino acid anilides. 0.975 g. (0.005 moles) of benzoyl-DL-alanine, or 0.894 g. (0.005 moles) of benzoylglycine, and 0.23 g. (0.0025 moles) of aniline were used/tube. The total volume was varied by the amount of citrate buffer solution (pH 5.0) added.

slightly larger yields than a 3-day incubation. The commercial sample employed (Difco papain, control No. 384352) catalyzed almost as well as a hydrogen sulfide-treated enzyme; much greater increases in yield resulted from use of a more concentrated buffer, and also from use of a higher ratio of aniline to benzamino acid. A similar experiment was set up for benzoyl-DL-phenylalanine which is, in contrast to benzoyl-

TABLE I  
*Effects of Conditions on Yield of Benzoyl-L-alaninanilide*

Buffer concentration	Aniline	Aniline/acid, molar ratio	Enzyme	Time	Yield
<i>M</i>	<i>mmoles</i>			<i>days</i>	<i>%</i>
0.10	25	1:1	Commercial	3	2.4, 2.7
0.10	25	1:1	Commercial	6	3.0, 3.6
1.00	25	1:1	Commercial	3	44, 46
1.00	50	2:1	Commercial	3	70, 71
1.00	100	4:1	Commercial	3	84, 85
1.00	25	1:1	Commercial	6	51, 52
1.00	25	1:1	H <sub>2</sub> S-treated <sup>a</sup>	3	48, 49
1.00	100	4:1	H <sub>2</sub> S-treated <sup>a</sup>	6	85, 86

<sup>a</sup> The hydrogen sulfide treated papain was processed according to the method of Grassmann (14).

alanine, relatively insoluble in the buffer mixture. The results were similar to those for the alanine derivative.

Another factor of importance in some anilide synthesis experiments is the type of substitution of the amino acid. Various derivatives of phenylalanine have been investigated; results are presented in Table II. These results point to a sharp specificity in reactivity between *N*-benzoyltyrosine and *O,N*-dibenzoyltyrosine when one operates at the usual pH "optimum." Under otherwise identical conditions, the dibenzoyltyrosine reacted rapidly, but the monobenzoyltyrosine was unreactive. The results first obtained in comparison experiments between the monobenzoyltyrosine and the dibenzoyltyrosine indicated

TABLE II  
*Reaction of Aromatic Amino Acid Derivatives With Aniline  
at pH 5.0 Under the Influence of Papain*

Acyl component	Yield of anilide from L-form %
<i>N</i> -Benzoyl-DL-phenylalanine	38.8, 40.2
<i>N</i> -Benzoyl-DL-tyrosine	0, 0
<i>N</i> -Benzoyl-L-tyrosine	0, 0
<i>O,N</i> -Dibenzoyl-DL-tyrosine	31.1, 32.5
<i>O,N</i> -Dibenzoyl-L-tyrosine	33.2, 34.3
<i>N</i> -Benzoyl- <i>p</i> -methoxy-L-phenylalanine	78.0, 79.0

From a 21-day incubation of 5.7 g. of *N*-benzoyl-DL-tyrosine and 3.75 g. of aniline, at 40° and pH 5.0, was recovered 5.5 g. of acylamino acid of m.p. 187-9°.

that the phenolic group must be masked for reaction to occur under the conditions employed. As a check upon this explanation, the methyl ether of *N*-benzoyl-L-tyrosine was prepared and tested. Here again reaction occurred readily.

The structural explanation of this specificity in behavior was found not to be fully valid, when a pH-activity curve was run for *N*-benzoyltyrosine as a substrate (Fig. 3). During the entire investigation various results emphasized the necessity for studying pH ranges in the anilide syntheses; the curves were accordingly plotted for the other phenylalanine derivatives. The results indicate a pH optimum of about 6.0-6.3 for the aromatic amino acids studied, and a pH value of about 5.0 for the aliphatic neutral amino acids, glycine, alanine, and valine.<sup>3</sup> Such

<sup>3</sup> A lower optimum of close to pH 4.0 has been recorded for carbobenzoxyglutamic acid by H. Wax and S. Melvin in this laboratory.

results may explain the change in pH optimum during papainolytic protein digestion (6). In most enzymic anilide experiments it has been customary to operate only near or at the pH usually considered to be optimal for the particular enzyme. This statement appears applicable to the report of the failure of chymotrypsin to catalyze the synthesis of benzoyl-L-tyrosinanilide (7). The results with benzoyltyrosine and aniline at pH values which are slightly above the "usual" optimum for papain, yet in a physiological range, emphasize the need for broader testing of such reactions, and for wider recognition of the variation in optimum with substrate.

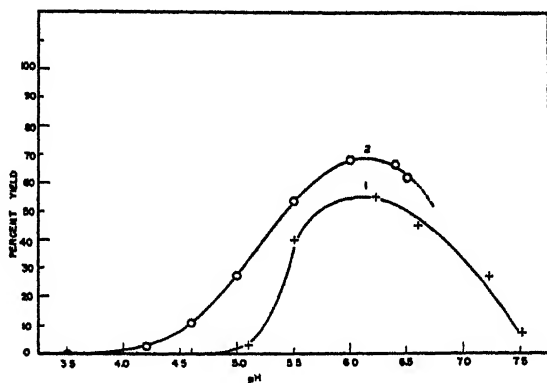


FIG. 3. Effect of pH on yield of *N*-benzoyl-L-tyrosinanilide, Curve 1; and *O,N*-dibenzoyl-L-tyrosinanilide, Curve 2.

The data in Table II, it may be noted, are fundamental to an enzymic resolution of tyrosine. Since tyrosine has been cheaply available as a by-product of commercial protein hydrolysis, a simple method of racemization, employed in this laboratory for several years (8), is also presented. The racemization of tyrosine with acetic anhydride was first announced by Bergmann and Zervas (9), but several discrete and relatively time-consuming steps were involved. A simple detail of critical utility is the carrying out of the combined acetylation and racemization steps at reflux temperature instead of in a boiling water bath. In conjunction with the resolution of formyl-DL-tyrosine by the use of brucine, several D-tyrosine derivatives have been conveniently prepared (8).

## EXPERIMENTAL

*Racemization of Tyrosine*

Thirty-six g. of technical grade tyrosine was refluxed on an oil bath with 120 ml. of technical grade glacial acetic acid and 30 ml. of technical 85% acetic anhydride for 1 hr. The mixture was concentrated under reduced pressure to a dense syrup. This was refluxed for 1 hr. with 200 ml. of 5 *N* hydrochloric acid solution.

The solution was again concentrated to a dense syrup, and treated with 100 ml. of water. The concentration was twice repeated with the addition of water, 5 g. of Darco G-60 was added, and the solution heated to boiling and filtered. The pH was brought to  $5.0 \pm 0.1$  with sodium hydroxide and the solution was allowed to stand overnight in the refrigerator. The dried precipitates weighed 31.5 g. (85% recovery). Other yields were 70%, 70%, and 85%. The color of the product was yellow, but the material was satisfactory as an intermediate in further processing. The product showed no rotation in the polarimeter.

*Acylamino Acids*

Hippuric acid was prepared by the method of Ingersoll and Babcock (10). Benzoyl-DL-alanine was made as previously described (11), in 85% yield. *N*-Benzoyl-DL-phenylalanine was made in the same manner as the glycine derivative. These substances were freed of contaminating benzoic acid by extraction of the crude, moist benzoylamino acid with boiling carbon tetrachloride. The *N*-benzoyl-*p*-methoxy-L-phenylalanine was prepared by the procedure of Behr and Clarke (12). The rotation observed for the methoxyphenylalanine was  $[\alpha]_D^{25} = -5.5 \pm 0.7^\circ$  for a 4.0% solution in 1 *N* HCl; for the benzoyl derivative it was  $[\alpha]_D^{25} = -3.8 \pm 1.0^\circ$  for a 4.0% solution in ethanol. The m.p. of the latter substance was 135–137°. The *N*-benzoyl-L-tyrosine and *N*-benzoyl-DL-tyrosine were made by hydrolysis of the benzoylated

TABLE III  
*Constants for Substances Not Found in the Earlier Literature*

Substance	M. P.	N, calcd.	N, found (micro Kjel- dahl)	$[\alpha]_D$
	°C.			
<i>O,N</i> -Dibenzoyl-DL-tyrosine	226–8	3.60	3.61	
<i>N</i> -Benzoyl-L-tyrosinanilide	208–8½	7.78	7.49	$-4.8 \pm 0.9^\circ$ at 24°C. <i>c</i> = 2.0 in chloroform
<i>O,N</i> -Dibenzoyl-L-tyrosin- anilide	243–4	6.03	5.95	$+17.5 \pm 0.2^\circ$ at 22°C. <i>c</i> = 4.0 in pyridine
<i>N</i> -Benzoyl- <i>p</i> -methoxy-L- phenylalaninanilide	223–4	7.49	7.31	$+16.2 \pm 0.4^\circ$ at 23°C. <i>c</i> = 4.0 in pyridine

esters in yields of 75% and 80%, respectively, from tyrosine (13), and the products had the m.p.'s previously recorded in the literature. *O,N*-Dibenzoyl-DL-tyrosine was prepared in 70% yield by the Schotten-Baumann procedure employing 3:2:1 moles of sodium hydroxide, benzoyl chloride, and DL-tyrosine, respectively.

A search of the literature failed to reveal a description of *O,N*-dibenzoyl-DL-tyrosine. The melting points and N analyses (microKjeldahl) are presented in Table III for this compound, along with the constants for anilides not previously reported.

The *O,N*-dibenzoyl-DL-tyrosine was prepared as follows: 48 g. of DL-tyrosine (0.267 moles) was dissolved in 400 ml. of 1 *N* NaOH, and 75 g. of benzoyl chloride (0.534 moles) and an additional 400 ml. of 1 *N* NaOH were added simultaneously at approximately equal rates to the tyrosine solution from dropping funnels. The reaction mixture was stirred vigorously and was cooled throughout the course of the reaction in an ice bath. The addition of benzoyl chloride required about 1 hr., and the mixture was then stirred for 0.5 hr. more. After acidification of the mixture with 250 ml. of 6 *N* HCl, a gummy precipitate formed and solidified upon cooling. This mass was broken up, washed with 200 ml. of water and dried in air. The resulting product was boiled for 10 min. with 400 ml. of carbon tetrachloride, filtered, and, after drying, the product was recrystallized from 3 l. of 50% ethanol. M. p. 222–225°C.; yield 74 g. (71%). The L-derivative was made in the same manner. M. p. 211–212°C.; yield 76%.

### *Anilide Synthesis*

All experiments were carried out using the same general procedure for adding the components of the incubation mixture and for obtaining and purifying the products of the reactions. Typically, 3 ml. of 3.0 *M* citric acid solution and 0.23 g. of redistilled aniline were added to 6.0 ml. of a 1 *N* sodium hydroxide solution containing the stated amount of benzoylated amino acid. To this was added 2.0 ml. of an enzyme solution prepared by dissolving 2.00 g. of commercial papain (either Difco control No. 384356 or Nutritional Biochemicals' Nos. 1953 or 1955) in 80 ml. of a 1.0 *M* citrate buffer solution (pH 5.0), filtering, and treating the filtrate with 2.00 g. of Merck's cysteine hydrochloride. The solution was brought to  $45 \pm 2$  ml. by use of the appropriate buffer solution. The pH was carefully adjusted to the indicated value with 10 *N* sodium hydroxide solution. The volume was brought to 50 ml. with 1.0 *M* citrate buffer of the proper pH and the incubation mixture was placed in 22 × 150 mm. stoppered tubes and incubated for 72 hr. at 40°C., with shaking by hand at approximately hourly intervals throughout the working day. The products were filtered, washed first with a small amount of water, then with two 10 ml. portions of 1 *N* sodium hydroxide solution (*N*-benzoyl-L-tyrosinanilide was washed with 2 *N* sodium carbonate solution instead) and then copiously with water, allowed to dry in the air for a minimum of 12 hr., and then weighed. In each case the melting points of the products obtained through this washing technique were in close agreement with the melting points of analytically pure samples.

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### SUMMARY

The effects of various experimental conditions upon the papain-catalyzed synthesis of peptide bonds have been studied in some detail. The factors studied have included citrate buffer concentration, aniline: acylamino acid ratio, volume of liquid employed for reaction, need for activation, time of reaction, and effects of substitutions in the side-chain of phenylalanine. Among critical factors, the effect of citrate buffer concentration is particularly important. The need for studying pH-yield relationships over a wide range of pH has been emphasized by the lack of activity at the usual pH of 5.0 for *N*-benzoyltyrosine, even though its optimum was found to be as close as 6.0. Esterification or etherification of the phenolic group of *N*-benzoyltyrosine has also been found to affect reactivity to a marked extent. Systematic studies of other conditions and combinations of conditions have been reported.

Data basic to enzymic resolution of alanine, phenylalanine, and tyrosine have been presented.

Several new anilides have been characterized.

The pH-yield curves for the enzymic formation of various benzoylamino acid anilides have been graphed.

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## Enzymic Synthesis of Peptide Bonds. II. "Preferences" of Papain Within the Monoaminomonocarboxylic Acid Series <sup>1</sup>

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### INTRODUCTION

In an earlier report (1), the effects of some reaction conditions upon the synthesis of acylamino acid anilides as catalyzed by papain-cysteine, were presented. The present work records a comparison of the effects of residues of a number of amino acids from within one group, the monoaminomonocarboxylic acid category. No such controlled comparisons appear previously to have been noted in the literature.

The incorporation of amino acid residues into anilides has shown for glycine, alanine, and leucine degrees of reactivity which appear to be consistent with the reactivity typically observed previously in separate experiments (2). No mention of the reactivity of valine has been found in the published anilide synthesis experiments of Bergmann; this amino acid has been investigated in the present study. When studied under comparable conditions (1) only small yields of the benzoylvalinanilide were found. Under otherwise identical conditions, considerable yields of benzoylglycinanilide were obtained, and the yields of benzoyl-L-alaninanilide and benzoyl-L-leucinanilide were much higher than for hippuric acid anilide<sup>4</sup> (Table I).

<sup>1</sup> Journal Paper No. J-1622 of the Iowa Agricultural Experiment Station, Project 1019. Some of the information in this paper was presented to the American Society of Biological Chemists, April 22, 1949, in Detroit, Michigan. This investigation was supported by Research Grant C-325 from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

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<sup>4</sup> Dr. Otto K. Behrens reports that carbobenzoxy-DL-penicillamine, which is structurally closely related to benzoyl-DL-valine, did not react at pH 5 with aniline, in the presence of papain-cysteine.

TABLE I

*Reactivity of Benzoyl Derivatives of Monoaminomonocarboxylic Acids  
With Aniline as Catalyzed by Papain at pH 5.0*

Benzoylated amino acid	Yield, % Papain Sample			
	No. 1	No. 2	No. 3	No. 3
Glycine	12, 12	14, 13	12, 11	10, 11
Alanine	46, 47	36, 39	40, 42	40, 36
Valine	3.4, 3.4	7.7, 9.5	6.2, 6.1	5.9, 5.9
Leucine	48, 50	57, 55	66, 65	57, 64

5.00 mmoles of each benzoyl DL-amino acid, and 5.00 mmoles of benzoylglycine were employed in each of these experiments. Sample No. 1 of papain was Difco papain, control No. 384352; No. 2 was Nutritional Biochemicals' control No. 1953; No. 3 was Nutritional Biochemicals' control No. 1955.

The relative nonreactivity of benzoylvaline at the usual pH of 5.0 led to testing the effect of pH variation. The results are given in Fig. 1. The product obtained was the expected benzoyl-L-valinanilide, and it can be seen that the yield is relatively small, attaining a maximum value at a pH between 5.0 and 5.5. This curve is similar to those for the corresponding glycine and alanine derivatives (1).

The behavior of benzoylated amino acids of the monoaminomonocarboxylic acid series was found to vary from virtual inactivity for

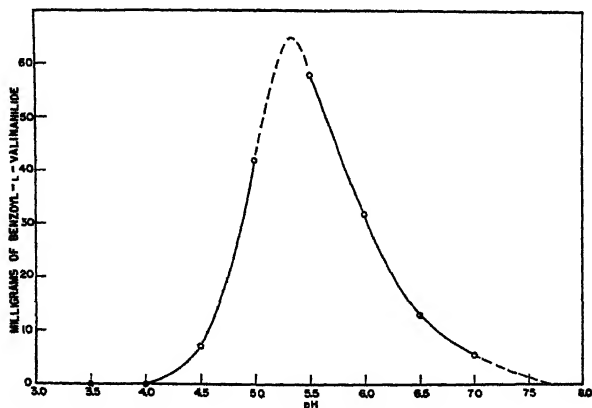


FIG. 1. Effect of pH on yield of benzoyl-L-valinanilide.

valine to semicomplete reactivity for leucine under otherwise identical experimental conditions. The term, "specificity," which has been applied in comparisons between, for example, basic and acidic amino acid residues in substrates (3), is inappropriate here. In accordance with these facts the term "preference" is employed instead. Preference, in this connotation, is to be distinguished from the type of preference previously noted in hydrolytic experiments (4), in which papain hydrolyzed one of two or more peptide bonds within the same substrate molecule. The preferences observed have been found to be on a kinetic basis.

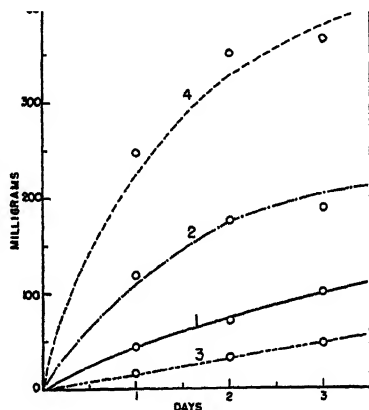


Fig. 2. Rate of synthesis of benzoylamino acid anilides. Glycine (Curve 1), alanine (Curve 2), valine (Curve 3), and leucine (Curve 4). Tubes were filtered at 24-hr. intervals. Curves represent plots of averaged results in duplicate tubes.

The importance of rates of reaction in determining preferences is illustrated by Fig. 2. which demonstrates that these syntheses had not attained equilibrium by the third day. Such biological significance as these studies have, needs to be considered against the background of the known dynamic nature of protein synthesis and the fact that in a biological system possessing lower concentrations of buffer, substrates, and enzyme, the approach to equilibrium would be theoretically far slower.

In further consideration of the preferences observed, it is helpful to consider the synthesis as occurring in two steps:

- a) Acylamino acid + aniline = acylamino acid anilide (soln.).
- b) Acylamino acid anilide (soln.) = acylamino acid anilide (ppt.).

One critical determinant of whether reaction (a) will proceed to give appreciable yield of anilide (others are enzyme-substrate interaction requirements, etc.) is whether or not the solubility of the particular anilide is less than that necessary to satisfy the equation:

$$K = \frac{(\text{anilide})}{(\text{acid})(\text{aniline})}$$

When it is less, the reaction proceeds in accordance with the original experimental observation (2). It is conceivable that in the synthesis of an anilide of limited yield, such as benzoyl-L-valinanilide, reaction (a) may proceed until enough acid and aniline are used up so that the solubility of the anilide is no longer less than the concentration required to maintain  $K$ . If this were a valid reason for the relative nonreactivity of benzoylvaline, the corresponding curve of Fig. 2 would become horizontal before the third day. Since it does not do so, the quantitative extent of the individual reaction is independent of the equilibrium solubility of the anilide.

Another conceivable way in which a property of the solid anilide would be determinant is that in which the solution becomes supersaturated with anilide and step (b) is rate-limiting. In order to check on this, the experiments of Fig. 2 were repeated with 200 mg. of anilide initially added to enough tubes so that daily filtration of the same tubes was unnecessary. In the event of supersaturation, precipitation would be most facilitated when in contact with added solid acting as seed. Since, within experimental error, no such enhancement was observed over the 3-day period, step (b) is not rate-limiting, and step (a) must be the rate-limiting one of the two. Step (a) is, of course, very likely a complex of reactions. Since (a) is the reaction that is enzyme-controlled, the extent of the overall synthesis, excluding the hypothetical exception given above, must be enzyme-controlled. This conclusion is borne out by other factors which have been studied; these appear to be predominantly of the kinetic type.<sup>5</sup>

DL-Valine has been found to inhibit growth of *Lactobacillus arabinosus* 17-5 to almost the same extent as does D-valine, even though L-valine lacks such activity (5). A related type of substrate behavior has been reported for benzoyl-DL-tyrosylglycinamide (6). In order to check on

<sup>5</sup> In other experiments in this laboratory, also, yields in this series of amino acids are different for ficin and for papain. Other acyl derivatives of valine and leucine also show similar enzyme preference for leucine.

whether the nonreactivity of benzoyl-DL-valine in the present study might result from a similar cause, benzoyl-L-valine was tested in companion experiments with benzoyl-DL-valine. The difference in the results of Table II was found to be insufficient to explain the limited rate of reactivity of benzoylvaline.

The validity of anilide syntheses as models of the biological synthesis of peptide bonds becomes worthy of more serious consideration as the result of these studies. In the absence of data for special enzymes for protein synthesis, and in the presence of data that the same enzymes catalyze both synthesis and hydrolysis under essentially similar conditions, the proteolytic enzymes deserve serious consideration as primary catalytic agents of protein synthesis (7). It may also be noted that D-amino acids (8) and antitrypsin (9) each inhibit both proteolytic enzymes and growth from amino acids. Since quantitative differences in

TABLE II

*Yield of Benzoyl-L-Valinanilide from Benzoyl-L-Valine and Benzoyl-DL-Valine*

Valine derivative <i>g.</i>	Yield %
0.27 benzoyl-L-valine	3.8
0.55 benzoyl-DL-valine	3.3

These experiments were carried out in the same way as those in Table I, except that half the molar quantities were used, in 16 × 150 mm. tubes.

anilide synthesis, furthermore, now are found to depend primarily upon enzymic behavior rather than upon anilide properties, such models are useful in clarifying this field of knowledge. Some chemical studies pertinent to both hydrolysis and synthesis are also more readily carried out with the simple acylamino acids than with peptide substrates as in hydrolysis experiments.

The present experiments are also of interest in relation to the observations of Roche and Mourgue (10). These workers observed a substantial liberation of leucine from casein during hydrolysis by papain, with little recovery of valine. They suggested that the leucine residues were located at readily accessible portions of the peptide chain, *i.e.*, near the ends, whereas valine would lie, according to this explanation, well within the peptide chains. Such an explanation will not, of course, explain the preferences observed in the synthesis of anilides. For anilide synthesis, the critical factor appears to be kinetically determined

enzyme preference rather than position of residues in substrates. In order to check further upon this relationship, the extents of hydrolysis of benzoylvalinamide and of benzoylleucinamide were also studied, since benzoylamino acid amides are standard papainolytic substrates (3). In experiments with these amides, the leucine derivative was hydrolyzed from 12–70%, depending upon conditions. In no corresponding case was the valine derivative hydrolyzed more than 4%. In the experiment of Table III, the conditions were nearly the same as for

TABLE III  
*Extent of Hydrolysis of Benzoylated Amino Acid Amides*

Amino acid	Hydrolysis %
Glycine	59
Valine	3.7
Leucine	32

These experiments were carried out in the same way as those in Table I. Ammonia from amide was determined by the Folin method (16) employing potassium borate as the alkaline agent.

the syntheses of Table I, except that this experiment deals with amides rather than anilides. A reasonable explanation covering casein, benzoylamino acid amides, and benzoylamino acid anilide formation, is that the rate of approach to equilibrium catalyzed by papain is much greater for leucine than for valine. That a position effect may also be of importance with leucine residues has been demonstrated in bacterial growth experiments with peptides of leucine (11,12).

## EXPERIMENTAL

### *Acylamino Acids*

The benzoyl derivatives of glycine, DL-alanine, DL-valine, and DL-leucine were prepared by the Schotten-Baumann reaction on each of the corresponding amino acids. The acid-precipitated products were freed of contaminating benzoic acid with hot carbon tetrachloride (8) while still moist. These derivatives gave the following melting points: hippuric acid 187½–9°C., benzoyl-DL-alanine 165–6°, benzoyl-DL-valine 129–31°, and benzoyl-DL-leucine 141–2°.

Benzoyl-L-valine was prepared by the reaction of 2.2 ml. of benzoyl chloride on 2.0 g. of L-valine ( $[\alpha]_D^{25} = +27.0^\circ$ ) dissolved in 40 ml. of 1 N sodium hydroxide solution. Acidification and recrystallization from carbon tetrachloride-hexane, ethyl acetate-hexane, and water, with mechanical separation of an oily fraction, finally gave well-formed crystals of melting point 131–2° and  $[\alpha]_D^{25} = +21.8 \pm 0.4^\circ$  for a

4.9% (wt./vol.) solution in 95% ethanol. Karrer and van der Sluys Veer prepared this compound by saponification of the ester (13) and reported a melting point of  $127^{\circ}$  and  $[\alpha]_D^{25} = +17.2^{\circ}$  in alcohol.

### *Benzoyl-L-Valinanilide*

In order to obtain workable quantities of this compound for analytical and reference purposes, 22 g. of benzoyl-DL-valine was shaken with 20 ml. of redistilled aniline in 1000 ml. of citrate buffer (1.0 M, pH 5.0). To this was added 1.0 g. of papain previously dissolved in 40 ml. of water, filtered, and treated with 1.0 g. of Merck's cysteine hydrochloride. The mixture was incubated at  $40^{\circ}$  for 72 hr. with occasional agitation. The resultant solid was filtered, washed with 20 ml. of 1 N sodium hydroxide solution, copiously with water, and dried in a desiccator. The product weighed 2.0 g. and had a melting point of  $219-21^{\circ}$ . It was dissolved in 20 ml. of hot dioxane, filtered free of a little colloidal impurity, treated with 20 ml. of water, and the mixture reheated almost to boiling. On cooling, 1.4 g. of anilide of melting point  $220-1^{\circ}$  was deposited.

Calcd.: N, 9.46; found: N, 9.41.

In a repetition employing three times as much enzyme, the yield was 7.5 g. of crude anilide after 3 days, and 12.6 g. after 10 days.

The specific rotation was determined on 452.8 mg. dissolved in chloroform and made up to 10.0 ml.

$[\alpha]_D^{25} = -80.6^{\circ} \pm 0.9^{\circ}$ .

### *Benzoylamino Acid Amides*

Hippurylamide was prepared by the method of Fischer (14).

The benzoylvalinamide and benzoylleucinamide were made by a modification of the procedure of Max (15) for the latter compound. Six g. of benzoylamino acid was covered with 14 ml. of acetyl chloride, chilled, and treated all at once with 5.5 g. of phosphorous pentachloride. This was shaken for 10 min. and then treated with 100 ml. of hexane. The precipitated acid chloride was suspended in 150 ml. of dry ether previously saturated with ammonia. Ammonia was then bubbled through the solution until no more precipitate appeared. The mixture was then filtered, washed with water, sodium carbonate solution, and finally recrystallized from water. This gave 3.0 g. of the leucine derivative, melting point  $169-70^{\circ}$ . The valine derivative, not previously described, had a melting point of  $220-1^{\circ}$ . Yield 2.1 g. Calcd.: N, 12.7; found: N, 12.6, 12.7.

### *Anilide Synthesis Experiments*

In the experiments typified by the set reported in Table I, the amino acid derivatives were treated in stoppered tubes ( $22 \times 150$  mm.) with 0.23 ml. (2.5 mmoles) of aniline and 12.0 ml. of citrate buffer (1.0 M, pH 5.0).

Two hundred mg. of papain was dissolved in 136 ml. of the same buffer solution, filtered, treated with 200 mg. of Merck's cysteine hydrochloride, and the volume was brought to 140 ml. with buffer solution. To each tube was added 17.0 ml. of this enzyme solution.

The tubes were incubated at  $40^{\circ}$  for 72 hr. with shaking by hand at 12 intervals. The products were filtered, each washed with two 10 ml. portions of 1 N sodium hydroxide

solution and two 10 ml. portions of water, allowed to dry in air overnight, and weighed. The alkaline wash has been found to be essential to ready purification, in many cases. The anilides obtained possessed m.p.'s, in close agreement with those in the literature. Mixed m. p.'s with analytically pure samples have given similar results.

The experiments on the effect of pH were run similarly. In this case the papain solution was prepared from 500 mg. of papain in 20 ml. of water, and treated with 500 mg. of cysteine hydrochloride after filtration. The amount of 1.0 *M* citrate buffer at each pH was 28 ml./tube, to which 1.0 ml. of the above enzyme solution was added. Since the components altered the respective pH's, the pH was carefully readjusted with concentrated sodium hydroxide solution or 3.0 *M* citric acid solution to the designated value, and then each volume brought to 30 ml. with the appropriate buffer solution.

#### ACKNOWLEDGMENTS

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#### SUMMARY

The comparative yields, under standard conditions, have been determined for four closely related benzoylamino acid anilides as catalyzed by papain-cysteine. The results emphasize differences not properly describable by the term, "specificity." "Preference" has been suggested as a more appropriate term.

The theory of enzymic anilide synthesis has been discussed. The occurrence or nonoccurrence of such reactions has been known to depend upon relative insolubility of the anilide (2). The relation of anilide solubility to the equilibrium of the reaction leading to its formation has been discussed. For those investigated syntheses that do occur to a measurable extent, the quantitative differences in the extents have been shown to depend primarily on the kinetics of the enzyme-controlled step. The significance of such experiments in an understanding of biological synthesis of peptide bonds has been discussed.

Rate curves illustrating preference for leucine, alanine, glycine, and valine, in that order, have been presented.

The amide and optically active anilide of benzoylvaline have been characterized.

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# Indoleacetic Acid Inactivating Enzymes from Bean Roots and Pea Seedlings<sup>1</sup>

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## INTRODUCTION

Tang and Bonner (1) have reviewed briefly the literature concerning auxin inactivation in plants and have described an indoleacetic acid inactivating enzyme from etiolated pea epicotyls. The enzyme was inhibited by potassium cyanide and carbon monoxide. It was very specific in its action and oxidized only indoleacetic acid; the enzyme was not active on indolepropionic acid, indolebutyric acid and a variety of other structurally related compounds. Tang and Bonner (1) suggested that this enzyme "may be an iron protein or even a heme-containing protein."

Experiments concerned with the effects of plant growth substances on bean plants revealed that an indoleacetic acid inactivating enzyme was present in the root sap of the yellow wax bean (2). This paper describes the properties of this indoleacetic acid inactivating enzyme from bean roots and reports a comparison of the properties of the enzymes from bean roots and etiolated pea epicotyls.

## I. THE INDOLEACETIC ACID OXIDIZING ENZYME FROM BEAN ROOTS

### MATERIALS AND METHODS

#### *Preparation of the Enzyme*

Sensation No. 2 yellow wax bean seeds were planted in pit sand in large clay pots, about 12 seeds/pot. The plants were supplied with Hoagland's (3) nutrient solution at weekly intervals and were watered as necessary with tap water. One to two-month old plants were used in these experiments.

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Bean roots were washed free of sand particles and organic matter with tap water, rinsed with distilled water, denodulated and blotted with paper towels to remove excess water. The roots then were ground in a glass mortar with white silica sand, and expressed by hand through a double layer of cheesecloth. The expressed juice was used as such without further treatment; the pH of the juice was in the range 6.4–6.6.

### *Manometric Measurement of Indoleacetic Acid Oxidation*

Enzymatic activity was determined by measuring oxygen uptake at 30°C. in a Warburg respirometer. The substrate (0.5 ml. of a solution containing the desired amount of neutralized indoleacetic acid) was placed in the sidearm; 0.15 ml. of 20% KOH and a filter paper wick were placed in the center well. The main compartment contained 1.0 ml. enzyme preparation, 1.0 ml. 0.1 *M* phosphate buffer pH 6.0 (0.1 *M*  $\text{KH}_2\text{PO}_4$  plus 0.1 *M*  $\text{Na}_2\text{HPO}_4$ ) and sufficient water to make a total volume of 3.0 ml. of reactants. When inhibitors were employed they were placed in the main compartment of the flask. Readings were taken during one hour.

## EXPERIMENTAL RESULTS

### *Presence of the Enzyme in Yellow Wax Bean Roots*

Oxygen uptake of whole bean root homogenate was greatly stimulated by the addition of indoleacetic acid, and was enhanced to a lesser extent by indolepropionic and indolebutyric acids (Fig. 1). The

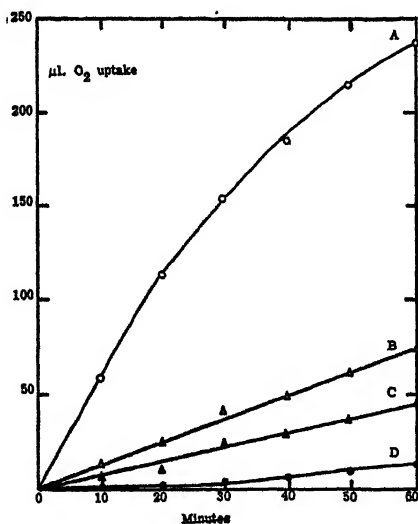


FIG. 1. Oxidation of various substrates by homogenate of bean roots. Curve A, 0.0125 *M* indoleacetic acid; curve B, 0.0125 *M* indolepropionic acid; curve C, 0.0125 *M* indolebutyric acid; curve D, oxygen uptake without added substrate.

neutralized (pH 6.8) ammonium salts of the acids were used. In all of our studies with other plant tissues which do not contain an enzyme oxidizing indoleacetic acid, a concentration of 0.001 to 0.01 *M* indoleacetic acid has proved inhibitory to their respiration; hence, it is logical to consider that the increased oxygen uptake observed here upon addition of indoleacetic acid results from the oxidation of the indoleacetic acid rather than from the stimulation of other respiration. Mitchell *et al.* (4) observed that increasing the concentration of indoleacetic acid increased the rate of oxygen uptake by bean root tissue but decreased the rate in the presence of an inhibitor (diethyldithiocarbamate) for the enzyme which oxidizes indoleacetic acid. (See section on concentration of substrate in Part II of this paper for data on this point with the enzyme from etiolated pea epicotyls.)

The large increases in oxygen uptake which were produced by the addition of the indole acids were attributed to enzymatic action, as boiled root homogenate plus these acids showed no such stimulation of oxygen uptake.

#### *Concentration of Substrate*

The rate of oxygen uptake varied with indoleacetic acid concentration from 0.00025 *M* to 0.025 *M*. It was maximum at substrate levels of 0.005 *M* to 0.01 *M*, and both higher or lower concentrations of indoleacetic acid gave lessened rates of oxygen uptake. Similar experiments conducted with indolepropionic and indolebutyric acids showed similar, but less pronounced, concentration optima. Each of the three acids at concentrations greater than 0.01 *M* supported lower rates of oxygen uptake than did 0.01 *M* solutions. This indicated an inhibition of enzymatic activity by excess substrate.

#### *Effect of pH*

The optimum pH was determined by adjusting portions of root homogenate and 0.1 *M* phosphate buffers to various pH's in the range pH 3.0–11.0 before mixing (initial pH), then mixing and measuring oxygen uptake; the final pH of the contents of the various flasks was measured after the oxygen uptake had been followed for 1 hr. Oxygen uptake was plotted against the average of the initial and final pH of the contents of the individual flasks. A rather sharp optimum was found in the region pH 6.0–6.5 (Fig. 2).

#### *Purification of the Enzyme from Bean Roots*

The enzyme was partially purified by fractionation with ammonium sulfate at 7°C. Table I shows the distribution of enzymatic activity between the precipitate and

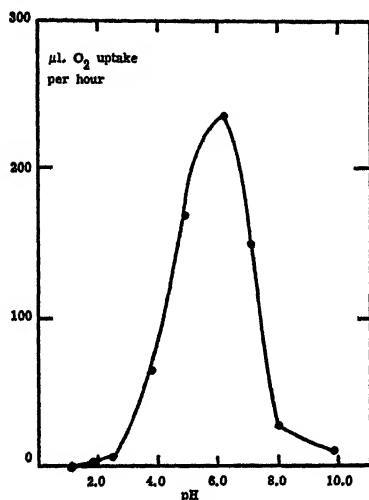


FIG. 2. Influence of pH on the rate of oxidation of indoleacetic acid by homogenate of bean roots.

supernatant at several concentrations of ammonium sulfate. A partial fractionation of bean root homogenate at levels of 10, 15, 20, and 25% saturation at 7°C. indicated that 20% saturation (1.09 *M*) was most effective for recovering maximum enzymatic activity in the supernatant with concomitant reduction of the respiration without added substrate.

The following procedure therefore was adopted: Crude root homogenate was 20% saturated with ammonium sulfate (14.4 g./100 ml.) at 7°C., allowed to remain in the cold for 2 hr., centrifuged for 15 min. at 560 × *g.* and the precipitate, which contained the bulk of the cellular debris and sand particles but very little enzymatic activity, was discarded. The supernatant liquid was taken to 75% saturation (54 g./100 ml.) with ammonium sulfate at 7°C., allowed to stand overnight at 7°C., and then centri-

TABLE I

*Distribution of Enzymatic Activity<sup>a</sup> in Centrifuged Salt-Fractionated Bean Root Homogenate*

Per cent saturation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> at 7°C.	Per cent of activity Supernatant	Precipitate
25 (1.37 <i>M</i> )	88	12
50 (2.74 <i>M</i> )	69	31
75 (4.10 <i>M</i> )	12	88
100 (5.47 <i>M</i> )	0	100

<sup>a</sup> Enzymatic activity was measured manometrically.

fuged for 20 min. at  $1100 \times g$ . The relatively inactive supernatant liquid, which contained soluble contaminants, was discarded and the precipitate, which retained most of the activity, was taken up in a small portion of distilled water and stored in the cold. No attempts have been made to purify the enzyme further as the preparation obtained by following the above scheme was capable of utilizing indoleacetic acid at a rapid rate and the respiration without added substrate was almost completely eliminated.

The enzyme is quite heat-labile. Root homogenate lost considerable activity upon standing for several hours at room temperature, but maintained almost full activity for at least a week when refrigerated.

### *Effect of Diluting the Enzyme*

Measurements of oxygen uptake with indoleacetic acid as substrate were made using undiluted and water-diluted enzyme preparations; both crude and salt-fractionated bean root homogenates were employed. In a typical experiment the precipitate obtained by 75% saturation of 30 ml. of crude bean root homogenate with ammonium sulfate at  $7^{\circ}\text{C}$ . was made up to 10 ml. with distilled water. This material contained 0.39 mg. protein nitrogen/ml. as compared to 0.48 mg. protein nitrogen/ml. in the crude material. Two-ml. portions were diluted 1:1, 1:3, 1:7, and 1:15 with distilled water. One-ml. portions were used in each Warburg flask. Figure 3 shows that at low concentrations of enzyme, the rate of oxygen uptake was proportional to enzyme concentration; at higher levels of enzyme, further increases in concentration were not accompanied by a proportionate rise in the rate of oxygen uptake. The data indicate the possible presence of an inhibitor in the enzyme preparations; such an inhibitor has been observed in pea plants by Tang and Bonner (5). There was no apparent "dilution effect" when either the crude or salt-fractionated enzymes were used.

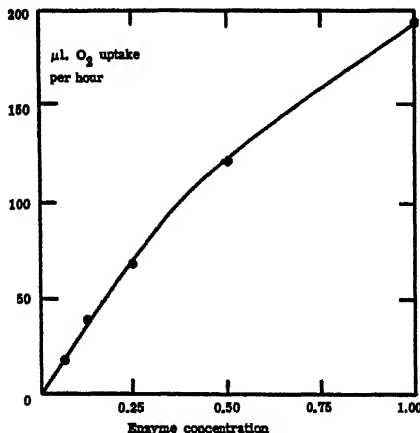


FIG. 3. Effect of enzyme concentration on the oxidation of  $0.006 M$  indoleacetic acid by salt-fractionated homogenate of bean roots.

*Activation of the Enzyme by Manganese*

The enzyme in bean roots was activated by the addition of manganese ion as  $\text{MnCl}_2$ . Total oxygen uptake of both crude and salt-fractionated enzyme preparations was increased by 50–100% by 0.0005  $M$  to 0.001  $M$   $\text{MnCl}_2$ . Concentrations of manganese greater than 0.001  $M$  gave less stimulation of oxygen uptake than did 0.001  $M$  manganese; lower concentrations, ranging downward to 0.0005  $M$ , stimulated oxygen uptake substantially. This activation by manganese appeared to be an effect on the enzyme, inasmuch as boiled enzyme plus indoleacetic acid plus manganese, or mixtures of indoleacetic acid and manganese with no enzyme showed no oxygen uptake.

The crude enzyme lost about one-third of its activity during dialysis at 7°C. overnight in a cellophane bag against distilled water. The original activity of the dialyzed crude homogenate was very nearly restored by the addition of 0.001  $M$   $\text{MnCl}_2$ .

A portion of crude homogenate was ashed, and the ash was taken up in concentrated  $\text{HCl}$  and neutralized to pH 6.5 with  $\text{NaOH}$ . This ash extract was added to the dialyzed crude preparation in an amount equal to that originally present. This procedure failed to restore any of the activity which had been lost through dialysis. That the ash extract contained no toxic or inhibitory materials was demonstrated by the fact that it produced no change in the rate of utilization of indoleacetic acid by crude homogenates when added in amounts equal to those added to the dialyzed material.

Salt-fractionated enzyme preparations were completely inactivated by dialysis in a cellophane bag against distilled water for 18 hr. at 7°C. Addition of 0.001  $M$   $\text{MnCl}_2$  restored 42–50% of the enzymatic activity as measured by oxygen uptake.

In a similar manner the action of other metallic ions was examined.  $\text{ZnCl}_2$  and  $\text{MgSO}_4$  at concentrations of 0.001  $M$  were ineffective in the restoration of activity to dialyzed, salt-fractionated bean root homogenate. Iron, as ferric citrate, and copper, as cupric sulfate, had essentially no effect on the activity of crude homogenates at 0.001  $M$  but caused 50% and 60% inhibition, respectively, at levels of 0.002  $M$ . There was no stimulation by copper as the concentration of cupric sulfate was reduced to  $5 \times 10^{-6}$   $M$ .

*Substrate Specificity*

Tang and Bonner (1) reported that the indoleacetic acid inactivating enzyme of etiolated pea epicotyls was highly specific for indoleacetic acid, and that it was not capable of utilizing indolepropionic acid, indolebutyric acid, and other structurally related compounds as substrates. Experiments with both crude and salt-fractionated bean root homogenate as enzyme source clearly established that indolepropionic acid and indolebutyric acid are used as substrates, but at a slower rate than indoleacetic acid. This was demonstrated by an increased oxygen uptake in their presence over that observed without added substrate. The rate of indolepropionic acid utilization was about 30% and of indolebutyric acid about 15% that of indoleacetic acid.

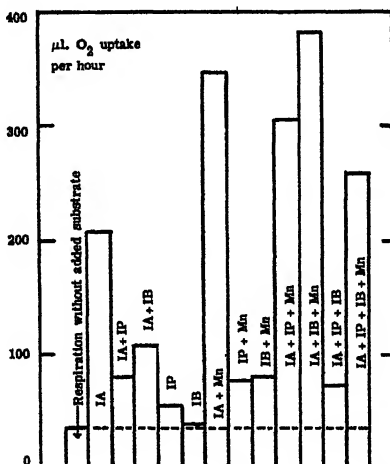


FIG. 4. Uptake of oxygen by crude homogenate of bean roots on various substrates singly and in combination, and the influence of manganese upon the oxidations. Each substrate, 0.006 *M* upon mixing;  $MnCl_2$ , 0.001 *M*. Abbreviations: IA, 3-indoleacetic acid; IP, 3-indolepropionic acid; IB, 3-indolebutyric acid.

An inhibition of indoleacetic acid utilization caused by indolepropionic and indolebutyric acids, either singly or in combination, has been noted. Figure 4 shows that the total oxygen uptake was less when a mixture of indolepropionic acid and indoleacetic acid was used than when indoleacetic acid alone was present. The same held true for a mixture of indolebutyric acid and indoleacetic acid, or a mixture of all

three acids. Indolepropionic and indolebutyric acids did not, however, completely suppress the utilization of indoleacetic acid, for oxygen uptake in all cases was greater when indoleacetic acid was present in combination with one of the other two acids than it was when either indolepropionic acid or indolebutyric acid alone was present. Because of the structural similarity of the three compounds this inhibition may well be competitive in nature. The initial concentrations of each of the acids upon mixing was 0.006 *M*. As mentioned earlier, the enzyme is inhibited by excessive substrate concentration, but the total concentrations of the acids used in this experiment were not sufficiently high to make it appear likely that the inhibitions observed were due to excessive substrate concentration. The inhibition was relieved by the addition of 0.001 *M*  $\text{MnCl}_2$ . Total oxygen uptake in the presence of added manganese in all instances was greater than the uptake obtained in the absence of any added manganese.

Indole is not used as a substrate; concentrations of 0.006 *M* and 0.012 *M* gave no stimulation of oxygen uptake over that observed without added substrate.

### *The Oxidative Nature of the Enzyme*

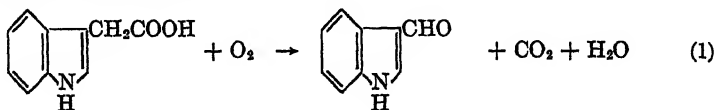
The respiratory quotient was determined by Warburg's direct method (6). The values for carbon dioxide evolution were corrected for bound carbon dioxide (Ref. 6, p. 18). Table II shows the data obtained in the oxidation of 0.003 *M* indoleacetic acid by crude bean root

TABLE II  
*R. Q. for Indoleacetic Acid Utilization by Bean Root Homogenate*

Reaction time	Total $\text{CO}_2$ evolved	Total $\text{O}_2$ consumed	R. Q.
<i>min.</i>	<i>μl.</i>	<i>μl.</i>	
10	54	49	1.11
20	116	104	1.11
30	150	140	1.07
40	175	163	1.08
50	185	178	1.04
60	198	192	1.03
75	214	207	1.03
90	220	217	1.02
105	227	224	1.01

homogenate. These R. Q. values very closely approximate unity and are in agreement with the findings of Tang and Bonner (1) on the indoleacetic acid inactivating enzyme in etiolated pea epicotyls. It is concluded that one mole of carbon dioxide is evolved/mole of oxygen consumed.

A possible reaction mechanism is:



Here indoleacetic acid is oxidatively converted to 3-indolealdehyde, with equimolar quantities of indoleacetic acid, oxygen, and carbon dioxide involved in the reaction. The product of the reaction is in fact a neutral substance, easily extractable with ether, showing carbonyl character, as exemplified by the formation of an insoluble 2,4-dinitrophenylhydrazone.

That the indole nucleus remains intact in the reaction was demonstrated by the development of a red color within 5 min. after Ehrlich's reagent, *p*-dimethylaminobenzaldehyde, was added to the ether extracted reaction products. A control of unreacted bean root homogenate treated in the same manner gave a yellow color, which is a negative test for the indole nucleus. Indoleacetic acid gave a weaker red color which was slower in developing. These results are in accord with the findings of Tang and Bonner (1) who showed by means of the Hopkins-Cole test that the indole nucleus remained intact in the reaction of indoleacetic acid with the pea enzyme.

At first it was considered possible that the enzymatic utilization of indoleacetic acid might involve an initial cleavage of the substrate molecule into an indole residue and acetate (or another two-carbon fragment) and an oxidation of the acetate. However, sodium acetate solutions of concentrations comparable to those of indoleacetic acid giving maximum oxygen uptake, proved to be slightly inhibitory, rather than stimulatory, to the respiration without added substrate. Apparently acetate, per se, plays no role in the oxidation of indoleacetic acid by the enzyme.

According to the postulated mechanism of the reaction (Eq. 1) the oxidation of 3.0 ml. of 0.003 *M* indoleacetic acid (9  $\mu$  moles) should require 202  $\mu$ l. of oxygen. When oxygen uptake by crude bean root homogenate plus 3.0 ml. of 0.003 *M* indoleacetic acid was plotted against time, a distinct break in the curve appeared at 50 min. The total oxygen uptake at this time was 206  $\mu$ l. or 102% of the theoretical

amount. In the presence of 0.001 *M*  $\text{MnCl}_2$  the total uptake in 90 min. with 18  $\mu$  moles of indoleacetic acid, corrected for respiration without added substrate, was 393  $\mu\text{l.}$  or 97% of the theoretical amount. Evidently manganese does not alter the course of the reaction but only serves as an activator.

### *Action of Inhibitors*

Table III summarizes the results of the action of certain inhibitors on the enzymatic oxidation of indoleacetic acid by bean root homogenate. The indoleacetic acid concentration was 0.006 *M* in all cases. The figures given represent the percentage inhibition of oxygen uptake in 30 min., and have been corrected for the amount of respiration without added substrate. The inhibitors were employed in aqueous solution; in the case of the Hecht-Eicholtz reagents and other acids, the neutralized sodium salts were used.

Inhibition by azide and cyanide suggested the enzyme is a metalloprotein. Sodium diethyldithiocarbamate, potassium ethyl xanthate, thiourea, 1-amino-8-naphthol-4-sulfonic acid and *o*-aminophenol-*p*-sulfonic acid (the latter two the so-called Hecht-Eicholtz reagents) are

TABLE III

*The Effect of Inhibitors on the Enzymatic Oxidation of Indoleacetic Acid by Bean Root Homogenate*

Inhibitor	Molar concentration of inhibitor				
	0.05	0.01	0.005	0.001	0.0005
	% inhibition				
Sodium azide	87	62	53	32	28
Sodium cyanide	100	100	100	100	100
Sodium fluoride	79	41	19	0	0
Sodium diethyldithiocarbamate	100	100	100	100	100
Potassium ethyl xanthate	100	100	100	100	100
Thiourea 0.1 <i>M</i> , 52	33	18	15	2	0
1-amino-8-naphthol-4-sulfonic acid	—	—	100	100	100
<i>o</i> -aminophenol- <i>p</i> -sulfonic acid	100	100	100	100	100
Ethylurethan 0.4 <i>M</i> , 15	—	—	—	—	—

There was no inhibition by iodoacetate, fluoroacetate, or malonate when the above concentrations (0.0005–0.05 *M*) were employed.

Enzymatic activity was measured manometrically.

all heavy metal reagents. Inhibition by these agents was taken as further indication of the presence of a metalloprotein. Sodium diethyldithiocarbamate, potassium ethyl xanthate and thiourea are known to inhibit the action of copper-containing enzymes such as tyrosinase and ascorbic acid oxidase (7,8). Sodium diethyldithiocarbamate is often considered a specific inhibitor for copper-containing enzymes (9). The strong inhibition by these three compounds suggested that the metal present in the enzyme or associated with it is copper. Tang and Bonner (1) concluded on the basis of inhibitor studies with cyanide and carbon monoxide that the indoleacetic acid inactivating enzyme in etiolated pea epicotyls contains iron.

Inhibition by ethyl urethan was probably of non-specific narcotic nature. Malonate caused no inhibition at concentrations of 0.0005 *M* to 0.05 *M*.

No inhibition of oxygen uptake by bean root homogenate was obtained in an atmosphere of 80% CO-20% O<sub>2</sub> compared with an atmosphere of air, or with 95% CO-5% O<sub>2</sub> compared with 95% N<sub>2</sub>-5% O<sub>2</sub>. This was true under conditions of normal light intensity, of very low light intensity (Warburg bath covered with a black cloth),

TABLE IV  
*The Action of Carbon Monoxide on the Enzymatic Oxidation of  
Indoleacetic Acid by Bean Root Homogenate*

Composition of gas mixture	Total O <sub>2</sub> uptake, $\mu$ l./hr., corrected for respiration without added substrate			% inhibition		
	(a) Low light in- tensity	(b) Normal daylight	(c) Dark- ness	(a)	(b)	(c)
80% CO-20% O <sub>2</sub>	255	290	87	None	None	None
Air	241	282	86	—	—	—
95% CO-5% O <sub>2</sub>	217	259	67	10	8	22
95% N <sub>2</sub> -5% O <sub>2</sub>	74	98	33	69	65	51

Different samples of plant material were used in carrying out these three experiments.

and in complete darkness. Carbon monoxide, generated from concentrated sulfuric and formic acids, was washed by passing it through alkaline 5% KMnO<sub>4</sub> and then through distilled water. The gas mixtures were introduced into the manometers and flasks by an evacuation technique (Ref. 6, p. 43). The data showing the lack of inhibition by carbon monoxide are given in Table IV. The apparent stimulation by carbon monoxide remains unexplained.

There was no inhibition by phenylmercuric nitrate or iodoacetate, which are mercapto inhibitors. Phenylmercuric nitrate, perhaps the more specific inhibitor,

exhibited no effect at concentrations of  $2.5 \times 10^{-4} M$  to  $4.1 \times 10^{-6} M$ , a concentration range in which it strongly inhibits certain enzymes containing active mercapto groups (10). Fluoroacetate likewise caused no inhibition.

The enzyme inactivating indoleacetic acid apparently is not present in the root nodules of the yellow wax bean, for indoleacetic acid at 0.006  $M$  to 0.0125  $M$  (concentrations giving strong stimulation of oxygen uptake by bean root homogenate) caused a marked (42–56%) inhibition of the endogenous respiration of bean root nodule homogenate. This effect was readily determined, as the nodule preparations possessed a high rate of endogenous respiration.

Bean root nodule homogenate was prepared as follows: excised nodules were rinsed in distilled water and blotted with paper towels, ground with silica sand in a glass mortar, and the sap expressed through a double layer of cheesecloth. The pulp was reground with a small portion of 0.1  $M$  phosphate buffer, pH 6.1, and the expressed liquid was combined with the first sap.

The enzyme apparently is not present to a measurable extent in the green leaves of the yellow wax bean, for 0.0125  $M$  indoleacetic acid had no stimulatory effect on the oxygen uptake of cell-free bean leaf sap. Tang and Bonner (5) observed that in several plant species the roots provided a better source of enzyme than did the tops of the plants.

## II. THE INDOLEACETIC ACID OXIDIZING ENZYME FROM ETIOLATED PEA EPICOTYLS

The indoleacetic acid inactivating enzyme present in the roots of the yellow wax bean was in many respects strikingly similar to the indoleacetic acid inactivating enzyme of etiolated pea epicotyls described by Tang and Bonner (1). There are, however, several obvious points of discrepancy in the properties reported for the enzymes from the two different plant sources. These properties have been examined carefully to determine whether the enzymes from bean roots and from etiolated pea epicotyls are truly different.

### METHODS AND MATERIALS

#### *Preparation of the Enzyme*

Pea seeds (var. *Alaska*) were soaked in water for 2 hr. and sown in pit sand. The seeds were germinated in complete darkness at 24°C. and 75% relative humidity. Epicotyls of 7-day old seedlings were used as the source of enzyme. These were ground in a cold room at 5°–7°C. with silica sand in a glass mortar; the juice, expressed through a double layer of cheesecloth, was kept under refrigeration until used or was lyophilized for later use (lyophilized whole cytoplasm, 120 mg./flask, took up oxygen at rates comparable to whole cytoplasm on a  $Q_{O_2}$  (N) basis). This scheme for the preparation of the enzyme is essentially the same as that outlined by Tang and Bonner (1), and for the sake of comparison their terminology, designating crude epicotyl juice as whole cytoplasm, will be used.

## EXPERIMENTAL RESULTS

*Presence of the Enzyme*

Whole cytoplasm prepared as outlined above from etiolated pea epicotyls contains the indoleacetic acid inactivating enzyme as shown by measurement of oxygen uptake. Into Warburg flasks were pipetted 1.0 ml. 0.1 *M* phosphate buffer pH 6.0, 1.0 ml. whole cytoplasm, and 0.75 ml. water in the main chamber; 0.25 ml. of a solution of the ammonium salt of indoleacetic acid of proper concentration in the sidearm; and KOH in the center well. Uptake of oxygen at 30°C. was recorded at 10-min. intervals.

The rate of oxygen uptake without added substrate by whole cytoplasm of etiolated pea epicotyls was higher (1 ml. sap, 60–120  $\mu$ l.  $O_2$ /hr.) than that of bean root homogenate (1 ml. sap, 40–60  $\mu$ l.  $O_2$ /hr.). Perhaps this was because the younger pea plants contained a greater abundance of endogenous substrates than did the mature bean plants, or perhaps this arose from inherent differences in the enzyme preparations derived from top and root tissues.

TABLE V

*Comparison of the Relative Rates of Indoleacetic Acid Oxidation  
by Enzymes from Bean Roots and Etiolated Pea Epicotyls*

	Bean root crude homogenate	$Q_{O_2}(N)$ Pea epicotyl whole cytoplasm
Respiration without added substrate <sup>a</sup>	79	18.5
Indoleacetic acid oxidation <sup>a</sup>	400	39
	25% supernatant <sup>c</sup>	20% supernatant <sup>c</sup>
Respiration without added substrate <sup>b</sup>	18	5
Indoleacetic acid oxidation <sup>b</sup>	856	125

Enzymatic activity was measured manometrically.

<sup>a</sup>  $Q_{O_2}(N)$  values based on total N determined by Semimicro Kjeldahl method.

<sup>b</sup>  $Q_{O_2}(N)$  values based on trichloroacetic acid precipitable nitrogen.

<sup>c</sup> Supernatant solutions from precipitation at 25% and 20% saturation with  $(NH_4)_2SO_4$  at 7°C.

Whole cytoplasm was salt-fractionated as described under purification of the bean root enzyme; the relative oxygen uptakes by whole cytoplasm and by the supernatant from whole cytoplasm brought to 20% saturation with ammonium sulfate (1.09 *M*) and centrifuged were compared with similar fractions of bean root homogenate. Salt-fractionation was effective in reducing materially the amount of respiration without added substrate. Table V shows that on the basis of activity/unit of total or protein nitrogen in either crude or salt-fractionated preparations, bean roots were a better source of enzyme than etiolated pea epicotyls.

*Concentration of Substrate*

The optimum substrate concentration was not as pronounced with pea epicotyl enzyme as with bean root enzyme; oxygen uptake was taken as a measure of enzy-

matic activity. At concentrations of indoleacetic acid between 0.00003 *M* (oxidation requires only 2  $\mu$ l.  $O_2$ ) and 0.0125 *M*, rates of oxygen uptake by the pea enzyme exceeded those without added substrate; but when the bean root enzyme was used, concentrations below 0.0006 *M* (oxidation requires 40  $\mu$ l.  $O_2$ ) did not support a rate of oxygen uptake exceeding that without added substrate. Apparently the stimulation of oxygen uptake by the enzyme from pea epicotyls cannot be explained entirely as an effect of indoleacetic acid acting as a substrate, although all the effects observed with the preparation from bean roots can be explained on such a basis.

### *Activation of the Enzyme by Manganese*

The effect of 0.001 *M*  $MnCl_2$  on oxygen uptake was variable; certain preparations of whole cytoplasm were stimulated whereas others were inhibited slightly. These variations are explainable as an effect of concentration, for lower concentrations of manganese, such as 0.0005 *M*, always stimulated crude whole cytoplasm. Whole cytoplasm lost about one-third of its activity during dialysis overnight in a cellophane bag against distilled water at 7°C. It was possible to restore the dialyzed enzyme to or above its original activity by the addition of 0.005 *M*  $MnCl_2$  (Table VI).

TABLE VI

*The Effect of Added Manganese upon the Rate of Indoleacetic Acid Oxidation by Crude and Dialyzed Whole Cytoplasm of Etiolated Pea Epicotyls*

	Oxygen uptake, $\mu$ l./hr. Crude whole cytoplasm	Dialyzed whole cytoplasm
Respiration without added substrate	51	18
Indoleacetic acid <sup>a</sup>	76	51
Indoleacetic acid plus 0.001 <i>M</i> $MnCl_2$ <sup>a</sup>	103	114

Enzymatic activity was measured manometrically.

<sup>a</sup> Values corrected for respiration without added substrate.

In a similar manner the action of other metals was examined.  $FeCl_3$ ,  $MgCl_2$ ,  $ZnCl_2$ , and  $CuSO_4$  at 0.001 *M* concentration produced no stimulation of oxygen uptake; cobalt, zinc, and copper were inhibitory when crude or dialyzed whole cytoplasm was used as the enzyme source.

### *Substrate Specificity*

When oxygen uptake by crude, dialyzed, and salt-fractionated whole cytoplasm was measured it was evident that the enzyme from etiolated pea epicotyls was capable of oxidizing indolepropionic and indolebuty-

ric acids. The rate of utilization diminishes with increasing length of the sidechain attached to the indole nucleus. Typical data for oxidation of these acids (0.0125 *M* concentration) by whole or salt-fractionated cytoplasm are shown in Table VII. When dialyzed whole cytoplasm was used, the indolepropionic acid was utilized at essentially the same rate as indoleacetic acid, but there was no corresponding change in the rate of use of indolebutyric acid.

TABLE VII

*The Oxidation of Indoleacetic Acid and Structurally Related Analogues  
by Enzyme Preparations from Etiolated Pea Epicotyls*

Substrate	Oxygen uptake, $\mu\text{l./hr.}$ , corrected for respiration without added substrate					
	Whole cytoplasm		Dialyzed whole cytoplasm		20% supernatant <sup>b</sup>	
	Uptake, $\mu\text{l. O}_2$	% of uptake on IA <sup>a</sup>	Uptake, $\mu\text{l. O}_2$	% of uptake on IA	Uptake, $\mu\text{l. O}_2$	% of uptake on IA
Indoleacetic acid	104	—	85	—	73	—
Indolepropionic acid	57	55	80	92	32	44
Indolebutyric acid	23	22	8	12	21	15

Enzymatic activity was measured manometrically.

<sup>a</sup> IA, 3-indoleacetic acid.

<sup>b</sup> Supernatant solution from precipitation at 20% saturation with  $(\text{NH}_4)_2\text{SO}_4$  at 7°C.

Measurement of oxygen uptake by the pea enzyme when two indole compounds were supplied together revealed a different response from that of the bean root enzyme. Either crude or dialyzed whole cytoplasm of pea epicotyls had a higher rate of oxygen uptake on a mixture of indole acids than the sum of the rates on the two acids supplied separately. Apparently the substrates were acting in a synergistic manner, whereas they had been mutually inhibitory to the enzyme from bean roots. Whole cytoplasm was inhibited by 0.001 *M*  $\text{MnCl}_2$  when only a single substrate was present, but was stimulated when the acids were used in combination as substrates; dialyzed whole cytoplasm was stimulated when the acids were used either singly or in combination as substrates. These results are summarized in Fig. 5.

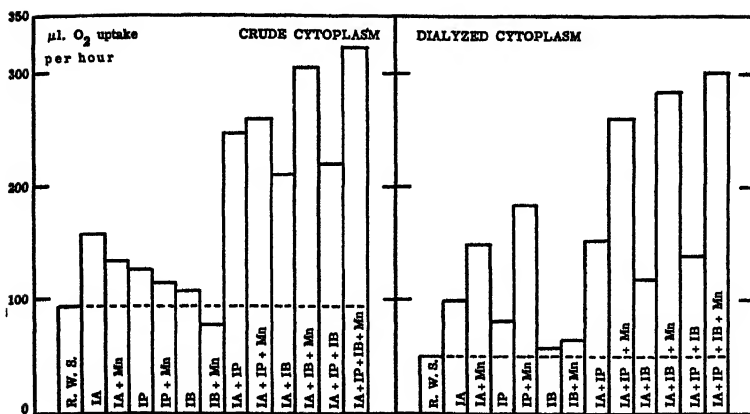


FIG. 5. Uptake of oxygen by crude and dialyzed cytoplasm from etiolated pea epicotyls supplied various substrates singly and in combination, and the influence of manganese upon the oxidations. Each substrate, 0.006 *M* upon mixing;  $MnCl_2$ , 0.001 *M*. Abbreviations: R. W. S., respiration without added substrate; IA, 3-indoleacetic acid; IP, 3-indolepropionic acid; IB, 3-indolebutyric acid.

### *Oxidative Nature of the Enzyme*

The R. Q. for indoleacetic acid oxidation by whole cytoplasm was approximately unity, thus confirming the work of Tang and Bonner (1) and agreeing with the value obtained using bean root homogenate. The R. Q. for indolepropionic acid oxidation was 0.2, which indicated that very little carbon dioxide was released. Apparently a different mechanism is involved in the enzymatic breakdown of the two acids. In an experiment of 5-hr. duration, undertaken to effect complete oxidation of indolepropionic acid at 0.003 and 0.005 *M* concentrations, there were no sharp inflections in the curves for oxygen uptake by crude or dialyzed whole cytoplasm. When indoleacetic acid is oxidized a rather sharp inflection occurs in the curve for oxygen uptake at the point representing exhaustion of substrate.

### *Action of Inhibitors*

The compounds which inhibited the action of the bean root enzyme were employed at the same concentrations and under similar conditions in experiments using crude and dialyzed whole cytoplasm of etiolated pea epicotyls. The inhibition by azide, cyanide, the Hecht-Eicholtz

reagents, sodium diethyldithiocarbamate, and thiourea was of the same order as had been observed with the bean enzyme. In addition a marked inhibition of oxygen uptake was caused by ethylene diamine and *o*-phenylenediamine at 0.001 *M*. With whole cytoplasm as the enzyme source, no inhibition of oxygen uptake was obtained with carbon monoxide under the same experimental conditions used previously in studies of the enzyme from bean roots.

The effects of several inhibitors on oxygen uptake by lyophilized whole cytoplasm (1) as the enzyme source were determined (Table VIII). Warburg flasks contained: 60 mg. dry wt. of lyophilized whole cytoplasm in 0.5 ml. volume; 1.0 ml. 0.1 *M* phosphate buffer, pH 6.0; inhibitor and sufficient water to make a total volume of reactants when mixed, of 2.5 ml. in the main compartment; 0.25 ml. 0.075 *M* indoleacetic acid in the sidearm (final concentration upon mixing 0.0075 *M*); and KOH in the center well. For tests of sulfide inhibition, distilled water was saturated with tank  $H_2S$  at 30°C.; suitable dilutions of the resulting 0.085 *M* solution were made with distilled water.

TABLE VIII

*The Effect of Inhibitors on the Enzymatic Oxidation of Indoleacetic Acid by Lyophilized Whole Cytoplasm of Etiolated Pea Epicotyls*

Inhibitor	Molar concentration of inhibitor				
	0.01	0.005	0.001	0.0005	0.0001
	% inhibition				
Hydroxylamine	83	76	67	47	—
8-Hydroxyquinoline	80	77	62	50	—
Thiourea	59	69	36	—	—
Potassium ethylxanthate	—	84	98	93	—
Sodium cyanide	—	—	43	38	5
Sodium azide	—	76	—	67	—
Sodium diethyldithiocarbamate	—	—	81	67	19
Hydrogen sulfide	—	—	48 <sup>a</sup>	—	14 <sup>b</sup>

Enzymatic activity was measured manometrically.

<sup>a</sup> 0.0017 *M*.

<sup>b</sup> 0.00017 *M*.

No inhibition of oxygen uptake by carbon monoxide was noted at levels of 76% CO–20%  $O_2$  with an air control, or at 91% CO–5%  $O_2$  with a mixture of 95%  $N_2$ –5%  $O_2$  as control; the Warburg apparatus was covered with a black cloth. These results are shown in Table IX. Although the rate of oxygen uptake compared with that in air was somewhat inhibited in the presence of 91% CO, this inhibition was not as great as that produced in the nitrogen–oxygen control. The inhibition in both instances was doubtless due to the reduced amounts of oxygen available to the enzyme.

TABLE IX

*The Action of Carbon Monoxide on the Enzymatic Oxidation of Indoleacetic Acid by Lyophilized Whole Cytoplasm of Etiolated Pea Epicotyls*

Composition of gas mixture	Total oxygen uptake  <i>μl./hr.</i>	Per cent inhibition compared with air control
76% CO-20% O <sub>2</sub>	97	None
Air	91	—
91% CO-5% O <sub>2</sub>	76	17
95% N <sub>2</sub> -5% O <sub>2</sub>	69	24

Enzymatic activity was measured manometrically.

Tank carbon monoxide (Matheson Co.) of 94% purity was used in these experiments. Of the 6% impurity in the carbon monoxide, 1.8% was oxygen. The maximum carbon monoxide concentration obtainable using this tank CO was 91%.

### *Colorimetric Method*

Inactivation of indoleacetic acid by etiolated pea epicotyl preparations was followed also by means of the colorimetric method of Tang and Bonner (1).

Whole cytoplasm is capable of rapidly inactivating indoleacetic acid. As reported by Tang and Bonner (1), there is no inactivation by whole cytoplasm which has been boiled previously for 5 min. Ten ml. of enzyme was added to 5 ml. of pH 6.6 phosphate-citrate buffer and 5 ml. of a solution containing 60 mg. indoleacetic acid/l. Samples were removed after incubation at 25°C. for 0, 1, 2, and 3 hr., and the reaction was stopped by boiling for 5 min. Colorimetric determinations were carried out on 2-ml. aliquots. The percentage inactivation was as follows: 1 hr., 20-40%; 2 hr., 76%; 3 hr., 100%.

### *Action of Inhibitors Measured with Colorimetric Tests*

As measured with the colorimetric method, inactivation of indoleacetic acid by the pea enzyme is inhibited by cyanide, azide, sodium diethyldithiocarbamate, and phenylthiourea. All reaction mixtures contained lyophilized whole cytoplasm (5.0 mg./ml.), pH 6.6 phosphate-citrate buffer, 15 mg. indoleacetic acid/l., and various concentrations of inhibitors. The mixtures were incubated 3 hr. at 25°C. The data are shown in Table X. Lyophilized whole cytoplasm was used in preference to crude juice because it produced less turbidity in the colorimeter tubes. No appreciable improvement of clarity of the mixtures resulted from centrifugation of the mixtures prior to mixing with the colorimetric reagent.

Tang and Bonner (1), on the basis of results with the colorimetric method for determining the inactivation of indoleacetic acid by lyophilized whole cytoplasm, have claimed that the enzyme is approxi-

mately 50% inhibited in the dark by carbon monoxide at a level of 95% CO-5% O<sub>2</sub>. Inasmuch as we had found no inhibition by carbon monoxide of indoleacetic acid oxidation by bean root homogenate or pea epicotyl cytoplasm as measured manometrically, the effect of carbon monoxide was examined under conditions similar to those used by Tang and Bonner (1).

TABLE X

*The Effect of Inhibitors on the Enzymatic Oxidation of Indoleacetic Acid by Lyophilized Whole Cytoplasm of Etiolated Pea Epicotyls*

Inhibitor	Molar concentration of inhibitor			
	0 005	0 001	0 0005	0 0001
	% inhibition			
Sodium cyanide	100	85	66	—
Sodium azide	91	—	84	—
Sodium diethyldithiocarbamate	—	100	100	100
Phenylthiourea	—	91	75	24

Enzymatic activity was measured colorimetrically.

Large Thunberg tubes were used as reaction vessels. (Tang and Bonner (1) used 50 ml. Erlenmeyer flasks equipped with vertical septa.) The main compartment of the Thunberg tube contained 50 mg. of lyophilized whole cytoplasm in 5 ml. liquid, 2.5 ml. pH 6.6 phosphate-citrate buffer, and 1.25 ml. distilled water. The sidearm of the tube contained 1.25 ml. of a solution of indoleacetic acid, 120 mg./l. The initial concentration upon mixing was 5.0 mg./ml. of enzyme and 15 mg./l. of indoleacetic acid. The Thunberg tubes were evacuated, filled with the various gas mixtures (tank CO was used and the gas mixtures were made up as described above), and the evacuation repeated four additional times to insure complete removal of the gases originally present in the tube. After closing, the tubes were equilibrated at 25°C., and the substrate was tipped from the sidearm. Two-ml. aliquots of reaction mixture were removed from replicate tubes for colorimetric analysis after incubation at 25°C. for 0, 1, and 3 hr.

Table XI shows that there was inhibition of enzymatic activity in the presence of carbon monoxide, but that this inhibition occurred both in light and in the dark. Furthermore, this inhibition was of the same magnitude as that obtained in the control atmosphere of 95% N<sub>2</sub>-5% O<sub>2</sub>. Evidently the inhibition arose from a lack of sufficient oxygen to allow for optimum enzymatic activity. Tang and Bonner (1) demonstrated the necessity of oxygen for enzymatic activity; in an atmosphere of argon they found no inactivation of indoleacetic acid by lyo-

philized whole cytoplasm, but the reaction proceeded smoothly in air or pure oxygen.

The role of oxygen in the enzymatic inactivation of indoleacetic acid was demonstrated further in experiments at various partial pressures of oxygen. Lyophilized whole cytoplasm was tested in Thunberg tubes as outlined above. Each tube contained 15 mg./l. indoleacetic acid initially upon mixing. Table XII summarizes the results; the data show that the rate of inactivation of indoleacetic acid is definitely a function of the  $pO_2$ .

*Action of Manganese on the Enzyme and on the Colorimetric Test*

The addition of  $MnCl_2$  to lyophilized whole cytoplasm was inhibitory to the action of the indoleacetic acid inactivating enzyme as measured colorimetrically. This inhibition varied from 80% with 0.0005  $M$   $MnCl_2$  to 15% with 0.00001  $M$   $MnCl_2$ . Manganese at the above concentrations does not affect the colorimetric determination.

TABLE XI

*The Action of Carbon Monoxide on the Enzymatic Oxidation of Indoleacetic Acid by Lyophilized Whole Cytoplasm of Etiolated Pea Epicotyls*

Composition of gas mixture	Time of incubation	Final amount Indoleacetic acid	Indoleacetic acid inactivated	Inhibition
	hr.	mg./l.	mg./l.	%
Air	0	15.0	0	—
	1	6.8	8.2	—
	3	3.1	11.9	—
95% $N_2$ -5% $O_2$ in light	0	15.0	0	—
	1	11.4	3.6	56
	3	10.4	4.6	61
95% $CO$ -5% $O_2$ in dark	0	15.0	0	—
	1	11.5	3.5	57
	3	10.6	4.4	63
95% $CO$ -5% $O_2$ in light	0	15.0	0	—
	1	11.6	3.4	59
	3	9.7	5.3	55

Enzymatic activity was measured colorimetrically.

TABLE XII

*Dependence of Enzymatic Oxidation of Indoleacetic Acid on the Partial Pressure of Oxygen in the Atmosphere*

Composition of gas mixture	Time of incubation	Final amount indoleacetic acid	Indoleacetic acid inactivated	Inhibition
	<i>hr</i>	<i>mg /l</i>	<i>mg /l</i>	<i>%</i>
Air	0	15.0	0	—
	1	9.0	6.0	—
	3	4.6	10.4	—
90% N <sub>2</sub> -10% O <sub>2</sub>	0	15.0	0	—
	1	9.4	5.6	7
	3	7.7	7.3	30
95% N <sub>2</sub> -5% O <sub>2</sub>	0	15.0	0	—
	1	12.4	2.6	57
	3	11.6	3.4	67
98% N <sub>2</sub> -2% O <sub>2</sub>	0	15.0	0	—
	1	13.8	1.2	80
	3	12.2	2.8	73

Enzymatic activity was measured colorimetrically.

Lyophilized whole cytoplasm was dialyzed in cellophane against running tap water for 20 hr. at 5°C. The inhibition by manganese was lessened thereby but not eliminated, nor was any stimulation apparent.

The exact nature of the stimulation of oxygen uptake by manganese is not known at present. The very apparent differences in the action of manganese upon the indoleacetic acid inactivating enzyme from bean roots and from etiolated pea epicotyls may arise from differences in the naturally occurring levels of manganese in the two tissues. Inasmuch as high concentrations of manganese are inhibitory to the enzyme from both sources, perhaps the optimum level is already present in the preparations of pea cytoplasm, whereas the manganese present in bean root homogenate requires supplementation.

## DISCUSSION

During an investigation of the effect of growth substances on the respiration of plants it was observed that a homogenate of the roots of wax beans rapidly oxidized 3-indoleacetic acid. Shortly thereafter the

report of Tang and Bonner (1) appeared describing the properties of a similar enzyme from etiolated pea epicotyls. As certain of the properties of the enzyme from the roots of wax beans differed from those reported by Tang and Bonner (1) for the enzyme from pea epicotyls, these properties were carefully checked.

Tang and Bonner (1) reported that the enzyme from etiolated pea epicotyls oxidized indoleacetic acid but not indolepropionic or indolebutyric acids. Our measurements on various preparations from etiolated pea epicotyls showed that indolepropionic and indolebutyric acids consistently stimulated oxygen uptake over that observed without added substrates. As with the preparations from bean roots, this stimulation was less than that observed with indoleacetic acid. Evidently both enzymes can attack each of the three substrates; their activity decreases with increasing length of the sidechain on the indole nucleus.

Tang and Bonner (1) stated: "The indoleacetic acid inactivating enzyme may be an iron protein or even a heme-containing protein. It does not in any case appear to be a copper protein of the type of polyphenol oxidase." This rather definitive statement was based upon the observation that the enzyme was inhibited by potassium cyanide and by carbon monoxide and that the carbon monoxide inhibition was light-reversible. Our tests with a number of inhibitors suggest that the enzyme more likely contains copper than iron. We have made tests under a variety of conditions but have been unable to demonstrate any inhibition by carbon monoxide. The results of Tang and Bonner (1) can be attributed in part (the reported reversal by light is not explained by our data) to a reduced rate of oxidation at the lower partial pressure of oxygen in their flasks containing carbon monoxide. They compared the rate of oxidation in air with the rate of oxidation in 95% carbon monoxide, 5% oxygen; they cited no reason for employing air as a control in such an experiment. In the absence of substantial evidence for light-reversible inhibition by carbon monoxide, little support remains for the argument that the enzyme contains iron or is a heme-containing protein.

The considerable activation of the enzyme by  $Mn^{++}$  is of interest. It is not clear how the  $Mn^{++}$  is working or what relation, if any, it has to the heavy metal (copper?) portion of the enzyme.

The enzymes from wax bean roots and from etiolated pea epicotyls are strikingly similar in their pH optima, respiratory quotients, end product of the oxidation of indoleacetic acid, response to inhibitors including carbon monoxide, substrate specificity, and response to

changes in the  $pO_2$ . The differences between the enzymes seem less striking than the similarities; the differences we have observed are in the amount of  $Mn^{++}$  required for stimulation, in the optimum substrate concentration, and in the mutual inhibition by substrates supplied in combination to bean root preparations, contrasted with the mutual stimulation in the oxidation of combined substrates by preparations from etiolated pea epicotyls.

We have been unable to demonstrate the presence of the indoleacetic acid inactivating enzyme in the green parts of bean plants, although its occurrence in etiolated pea epicotyls demonstrates the potential ability of plant tops to produce it. Tang and Bonner (5) have attributed the lack of activity in tops to light-destruction of the enzyme and to the presence of an enzyme inhibitor. Although the physiological role of the enzyme which inactivates indoleacetic acid is not entirely clear, its presence in roots could be interpreted as serving to foster active root growth by destroying the indoleacetic acid which inhibits root growth. In etiolated plants, where elongation of tops is strongly stimulated by indoleacetic acid, the enzyme may serve to limit the rate of growth.

#### ACKNOWLEDGMENTS

We wish to acknowledge the helpful direction and suggestions of A. J. Riker and T. C. Allen.

#### SUMMARY

The properties of the indoleacetic acid inactivating enzymes from roots of yellow wax beans and from etiolated pea epicotyls have been compared.

The enzymes exhibited similarity in pH optima, respiratory quotients in the oxidation of indoleacetic acid, nature of the products formed, stimulation by manganese, and response to various inhibitors. Both enzymes are inhibited by heavy-metal reagents; they are not inhibited by high concentrations of carbon monoxide.

Apparent differences were observed in optimum substrate concentration; in the utilization of mixtures of indoleacetic, indolepropionic, and indolebutyric acids; and in the relative amounts of manganese required for stimulation of enzymatic activity.

The questions of substrate specificity, nature of the metal present in the enzyme, and carbon monoxide inhibition were carefully examined

because of discrepancies between our observations and reports in the literature. The following results from our experimental work are at variance with earlier reports (1) concerning the enzyme: (a) The enzyme from each plant source is active on indolepropionic acid and indolebutyric acid in addition to indoleacetic acid. (b) Tests with inhibitors have suggested that the metal present in the enzyme is copper. (c) The enzymes are not inhibited by carbon monoxide, but enzymatic activity is affected by the partial pressure of oxygen.

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# The Anomalous Heat Inactivation of *Clostridium perfringens* Lecithinase

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## INTRODUCTION

That certain substances of biological origin exhibit anomalous heat inactivation has been known since Arrhenius (1) stated that staphylococcal hemolysin was more greatly inactivated by heating at 70°C. than it was by heating at 100°C. A similar phenomenon has been reported for certain enzymes of *Clostridium perfringens* by Guillaumie *et al.* (2) and for the lecithinase of *Bacillus cereus* by Chu (3). In general, when such materials were heated at 100°C. for a few minutes, after having been inactivated by heating at a lower temperature, partial activation occurred.

We encountered this phenomenon while investigating the lecithinase of *Cl. perfringens* and deemed it of interest to investigate some of the factors affecting this irregular heat inactivation.

## MATERIALS AND METHODS

The strain of *Cl. perfringens* used in this study was grown in 3-l. lots in a medium similar to that described by Adams and Hendee (4), containing about 3% pancreatic digest of casein, 0.5% insoluble dextrin, 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.5%  $\text{K}_2\text{HPO}_4$ . The cultures were incubated in a 39°C. water bath for 5-6 hr. The hydrogen-ion concentration was kept between pH 6.0 and 6.5 by frequent adjustment with 5 *N* NaOH. When growth had ceased, as shown by diminution of gas production and the cessation of acid formation, the culture was centrifuged and the supernatant fluid was saturated with ammonium sulfate. After 1 hr., the scum that rose to the surface was removed, dissolved in approximately 100 ml. of distilled water, and dialyzed against several changes of distilled water in the cold. The dialyzed material was centrifuged, the precipitate discarded, and to the supernatant fluid was added 40 g. ammonium sulfate/100 ml. This material was again centrifuged, the precipitate was taken up in a small volume of water and dialyzed against distilled water in the cold to free the solution of ammonium sulfate. This material will be referred to as "crude lecithinase."

Some preparations of crude lecithinase were frozen and dried and combined for further purification by a procedure similar to that of van Heyningen and Bidwell (5). Two grams of the frozen and dried material was dissolved in 20 ml. of water, the pH was adjusted to 6.0–6.2 and the solution was clarified by centrifuging. The supernatant fluid was chilled in crushed ice, and to it was added 0.2 g. of purified yeast nucleic acid dissolved in 5 ml. water and adjusted to pH 7.0. The pH of the chilled mixture was adjusted to 4.5 with 1.7 *N* acetic acid. The mixture was centrifuged in the cold, the precipitate was redissolved in 20 ml. water and the pH was adjusted to 6.5. A saturated solution of protamine (pH 6.5) was added until no further precipitate was formed, the mixture was centrifuged and the supernatant fluid removed. To it was added 4 g. of ammonium sulfate, the sediment obtained after centrifuging was discarded, and 12 g. of ammonium sulfate was added to the supernatant fluid. After centrifuging, the precipitate was taken up in 5 to 6 ml. water and stored in the cold under toluene. This material will be referred to as "partially purified lecithinase." It contained about 2000 egg units/ml.

Lecithinase determinations were performed by the method of van Heyningen (6). A Klett colorimeter was used to determine turbidity. Activity is expressed in egg units (E.U.).

Dilutions of stock lecithinase solutions were made in 0.1 *M* borate buffer, pH 7.6, unless otherwise indicated. They were heated in 1.0-ml. portions in stoppered pyrex tubes, 12 × 100 mm. Timing was started when a control tube containing 1 ml. water reached the desired temperature.

## EXPERIMENTAL

One-milliliter portions of dilutions of crude lecithinase were heated at 50, 60, 70, 80, 90, and 100°C. for 10 min. and were then tested for activity. The data, represented in Fig. 1, indicate that heating at temperatures higher than 60–70°C. inactivated less enzyme than heating at these temperatures, and also that the greater the dilution of the enzyme, the less the inactivation.

The data in Fig. 2 indicate the effect of hydrogen-ion concentration from pH 5.0 to pH 9.0 on inactivation by heat. The enzyme solution contained 27 E.U./ml. Below pH 7, 0.1 *M* acetate buffers were used; above pH 7, borate buffers were used.

Since there seemed to be some relation between the concentration of the enzyme and the amount of inactivation by heating, it was thought that the enzyme might be associating to form an inactive complex, probably bound through some divalent ion. Calcium or magnesium are the only ones that might be expected to be present in appreciable amounts in the casein digest culture medium and in the crude lecithinase. It seemed quite unlikely that heat denaturation could be responsible for the inactivation of the enzyme when heated at the lower

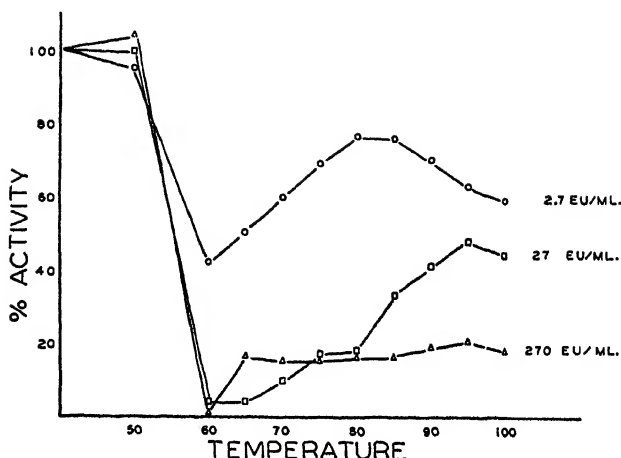


FIG. 1. Inactivation of lecithinase of *Cl. perfringens* by heat.

temperatures, for this would involve the absurdity of postulating that heat denaturation could be reversed by heating at higher temperatures.

A sample of crude lecithinase was electrodialedyzed to free it, so far as possible, of easily dissociable ions. During the process, it was necessary to adjust the pH of the material within the cell with 1 *N* ammonium hydroxide to maintain the pH above 5.0. The enzyme solution, after electrodiaalysis, was diluted to 16 E.U./ml., and  $\text{CaCl}_2$  and  $\text{MgCl}_2$  were added to separate portions. Enzyme activity of these solutions after

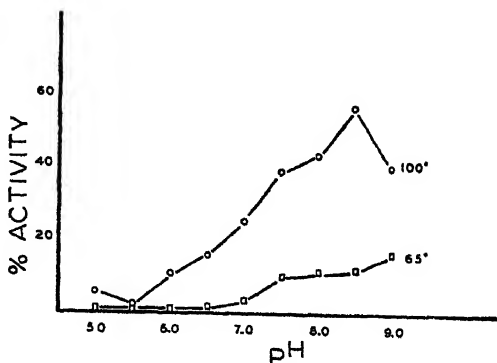


FIG. 2. Effect of hydrogen-ion concentration on heat inactivation of lecithinase of *Cl. perfringens*.

heating 10 min. at 65°C., 10 min. at 100°C., and 10 min. at 65°C. followed by 0.5 min. at 100°C. are given in Table I. It is apparent that the anomalous heat inactivation was almost entirely abolished by freeing the enzyme of easily dissociable ions, that this phenomenon was restored by the addition of  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$ , and that  $\text{Ca}^{++}$  was more effective than  $\text{Mg}^{++}$  in this regard.

TABLE I  
*Effect of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  on the Heat Inactivation of Crude  
*Cl. perfringens* Lecithinase*

Material added	Original activity			
	No heat	65°C. for 10 min.	100°C. for 10 min.	65°C. for 10 min. + 100°C. for 0.5 min.
None	% 100	% 75.4	% 52.5	% 80.2
0.001 M $\text{Ca}^{++}$	100	60.8	48.2	65.0
0.01 M $\text{Ca}^{++}$	100	15.6	36.2	24.4
0.1 M $\text{Ca}^{++}$	113 <sup>a</sup>	6.9	8.7	15.6
0.001 M $\text{Mg}^{++}$	109	77.0	51.8	70.6
0.01 M $\text{Mg}^{++}$	96.9	44.4	45.6	43.8
0.1 M $\text{Mg}^{++}$	96.9	19.4	49.4	36.3

<sup>a</sup> This value is falsely high because of turbidity not associated with lecithinase activity.

When dilutions of partially purified lecithinase were heated at 65 and 100°C., the inactivation of the enzyme was no longer anomalous, as the data in Table II indicate. Only in the most concentrated solution was there greater inactivation at 65°C. than at 100°C. It is probable that the purification of the lecithinase reduced the concentration of divalent ions to a point insufficient to allow anomalous inactivation, for, as will be seen, the addition of  $\text{Ca}^{++}$  to the partially purified enzyme restored the anomalous inactivation. Surface denaturation may, in some measure, account for the greater inactivation of the dilute solutions; Macfarlane and Knight (7) reported that *Cl. perfringens* lecithinase underwent surface denaturation easily, and we found that a solution of partially purified lecithinase containing 2 E.U./ml., through which hydrogen bubbled for 1 hr., lost about 75% of its activity. A similar solution shaken for 1 hr. while exposed to the atmosphere lost about 68% of its activity.

It was noted in these experiments that perceptible opalescence often appeared on heating and that, in general, the inactivation of the enzyme paralleled, roughly, the amount of opalescence. It was thought that if complex-formation did take place, it should be possible to centrifuge out some of the complex. Partially purified lecithinase (85 E.U./ml.) was made 0.01 *M* with  $\text{CaCl}_2$ , heated at 65°C. for 10 min. and centrifuged for 10 min. at 35,000 r. p. m. in a multispeed head (International Equipment Co.). The sediment was suspended in borate buffer, a sample removed for the determination of lecithinase, and the remainder was heated for 10 min. at 100°C. A portion of enzyme solution which had not been heated at 65°C. was treated similarly. As can be seen from the data in Table IIIA, the heating at 65°C. inactivated almost all of the enzyme. The enzyme activity of the suspended precipitate increased appreciably on being heated at 100°C. for 10 min.

TABLE II  
*Effect of Concentration of Enzyme on Inactivation by Heat  
of Partially Purified Cl. perfringens Lecithinase*

E.U./ml.	Original activity	
	65°C. for 10 min. %	100°C. for 10 min. %
400	12.5	24.7
200	26.7	23.5
100	45.0	33.9
50	56.0	35.8
25	60.6	30.2
12.5	76.8	20.1
6.25	63.2	22.6
3.12	35.3	2.4
1.56	16.6	<2

This experiment was repeated with another solution of partially purified enzyme (Table IIIB). The heating at 100°C., however, was for 0.5 min. A sample was removed for the determination of enzyme activity and the remainder of the solution was recentrifuged. It is apparent that the complex formed at 65°C. was sedimentable, that a large measure of enzyme activity was restored by this short heating at 100°C., and that the enzyme was no longer sedimentable after this activation. Specimens of partially purified lecithinase which had not been heated at 65°C. exhibited little loss of activity from the supernatant fluid on centrifuging. The slight activity found in the small amount

of sediment was not increased appreciably by being heated for either 0.5 or 10 min. at 100°C.

From these data, it appears probable that complex-formation did take place at 65°C., and that the complex was dissociated at 100°C., with the consequent freeing of the enzyme. With regard to the mechanism of complex-formation it is suggested that the lecithinase molecules were reversibly linked through calcium or magnesium and that the complex was disaggregated when heated at 100°C. by the formation of an insoluble calcium compound—probably one of the phosphates. It seems likely that the mechanism of the anomalous inactivation of the lecithinase of *Cl. perfringens* is not identical with that proposed by Hofstee (8) for the inactivation of urease at low temperatures. In the latter case, it appeared that the enzyme molecules tended to associate with each other or with other protein molecules directly, and that no divalent ions were involved.

TABLE III

*Sedimentation of Heat-Inactivated Cl. perfringens Lecithinase*

	Original activity	
	A %	B %
Enzyme heated at 65°C., centrifuged		
Supernatant fluid	2.8	1.3
Resuspended sediment before heating at 100°C.	0.19	0.40
Resuspended sediment after heating at 100°C.	10.1	44.3
Resuspended sediment centrifuged after heating at 100°C.		
Supernatant fluid		35.0
Sediment		0.96

From the results of this study it appears that the determination of the inactivation by heat of substances which can undergo complex-formation is a rather complicated problem, particularly if they also undergo surface denaturation in dilute solution. Such determinations should be made only on highly purified solutions, whose content of inorganic ions is definitely known.

#### SUMMARY

1. The anomalous heat inactivation of *Cl. perfringens* lecithinase is probably due to complex-formation.
2. The formation of this complex may involve interaction of lecithinase with calcium or magnesium ions.

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# A Comparison of the Provitamin A Activity of $\beta$ -Carotene and Cryptoxanthin in the Chick

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## INTRODUCTION

The provitamin A activity of all-*trans*-cryptoxanthin,  $C_{40}H_{55}OH$ , has generally been considered to be about 50% that of all-*trans*- $\beta$ -carotene,  $C_{40}H_{56}$ . Previous investigations from these laboratories have reported that all-*trans*-cryptoxanthin has 56% (1) and 60% (2) of the provitamin A activity of all-*trans*- $\beta$ -carotene in the rat. On the other hand, With (5), using yellow corn in the diet as the source of cryptoxanthin, has calculated the biological action as about double that of  $\beta$ -carotene based on growth-promoting effect on chicks. With and Wanscher (6) on the basis of percentage of storage of vitamin A believe that the effectiveness as compared with  $\beta$ -carotene is even greater. From these results, With concluded that cryptoxanthin must act directly, since it would be expected to produce only about one-half of the activity of  $\beta$ -carotene if it had to be converted to vitamin A before exerting its physiological effect.

Johnson, Swick and Baumann (4) have failed to confirm some findings of With; their results with chicks showed that, on the basis of growth, cryptoxanthin has about 75% of the activity of  $\beta$ -carotene and approximately equal value when tested by the storage of vitamin A in the liver.

Because of these conflicting reports on the potency of cryptoxanthin

<sup>1</sup> Nee Mookerjee; Fellow of the Indian Government.

<sup>2</sup> Paper No. 228 of the Department of Biochemistry and Nutrition, University of Southern California. The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation.

<sup>3</sup> Contribution No. 1323.

in the chick, the present study was undertaken. A determination of the optimum duration of the test and dosage for obtaining a straight line curve for log-dose versus growth in chicks was also made using  $\beta$ -carotene.

### METHODS

The procedures used were similar to those reported previously (3). White Leghorn roosters from the same source as employed earlier were used for the tests. Diet 2 was fed during the entire depletion and assay periods. The length of the depletion period was 21 days in both series of tests reported below. At that time all too-light or too-heavy chicks were culled and the remaining ones were equally distributed between the assay and control groups. The chicks were identified by a leg band assigned to each at the end of the depletion period.

Supplements were fed daily in 0.1 ml. of Wesson oil containing 0.5% of  $\alpha$ -tocopherol (0.5 mg./dose). In Ser. I,  $\beta$ -carotene was fed at four levels, *viz.*, 1.0, 2.0, 3.0, and 4.0  $\mu$ g. daily; while in Ser. II, only the lower three groups were used. Cryptoxanthin was fed in both series at levels of 1.75, 2.75, or 3.75  $\mu$ g. daily. In both series, a negative control group, which received only the Wesson oil containing  $\alpha$ -tocopherol, and a positive control group, which were fed 95 U. S. P. units of vitamin A daily in the form of natural ester, were included. Basal diet and water were supplied *ad lib*.

The pigments used were stored in brown bottles in a deep freeze and the samples were changed twice weekly. Spectrophotometric analysis showed no appreciable loss in potency of  $\beta$ -carotene or cryptoxanthin on standing in Wesson oil in the deep freeze during the entire assay period.

### RESULTS AND DISCUSSION

The experimental results are given in Table I. The growth response of chicks receiving four levels of  $\beta$ -carotene in Ser. I is plotted against log-dose in Fig. 1.

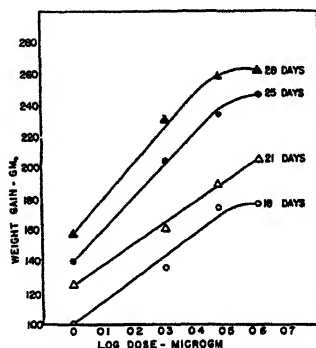


FIG. 1. Relationship of gain in weight in chicks to log of daily dosage of  $\beta$ -carotene on the 18th, 21st, 25th, and 28th days, respectively.

TABLE I

*Summary Table Giving the Average Gain in Body Weight of Roosters Receiving  $\beta$ -Carotene and Cryptoxanthin in Cottonseed Oil Containing  $\alpha$ -Tocopherol, or Cottonseed Oil Alone (Negative Controls), or Excess Vitamin A Ester (Positive Controls), Daily*

Supplement	Dose/day ( $\mu$ g)	Starting body weight (g)	Average gain in weight, g, on following assay days <sup>a</sup>				Potency ( $\beta$ -carotene = 100)
			18(I) 19(II)	21(I) 21(II)	25(I) 23(II)	28(I) —	
Ser. I. 16 birds/group. Average weight at start of depletion, 36 g.							
All- <i>trans</i> - $\beta$ -carotene	1.0	169	101(12)	126(10)	141(10)	159(10)	—
	2.0	167	139	163(15)	204(15)	232(15)	—
	3.0	168	175	191	234	259	—
	4.0	168	177	206	247	263	—
All- <i>trans</i> -cryptoxan- thin	1.75	169	120(13)	124(13)	146(12)	164(10)	55
	2.75	169	126(15)	139(15)	161(15)	201(14)	46
	3.75	170	172(13)	187(13)	235(13)	252(13)	76
Positive controls	95 <sup>b</sup>	168	226(15)	242(15)	311(15)	338(15)	—
Negative controls	0.0	170	(2)	(1)	(1)	0	—
Ser. II. 18 birds/group. Average weight at start of depletion, 38 g.							
All- <i>trans</i> - $\beta$ -carotene	1.0	168	138(17)	155(17)	171(17)	—	—
	2.0	165	163	175	191	—	—
	3.0	170 <sup>c</sup>	188	196	215	—	—
All- <i>trans</i> -cryptoxan- thin	1.75	169	132(15)	140(15)	149(15)	—	53
	2.75	170	156	171	186	—	56
	3.75	171 <sup>d</sup>	167	183	211	—	54
Positive controls	95 <sup>b</sup>	170	198	212	240	—	—
Negative controls	0.0	171	(7)	(7)	(6)	—	—

<sup>a</sup> The figures in parentheses represent the number of birds surviving in each group of which the average weight is given.

<sup>b</sup> U. S. P. units of vitamin A.

<sup>c</sup> Only 17 birds in this group.

<sup>d</sup> Only 16 birds in this group.

There is an excellent proportionality between the gain in weight and log-dose of  $\beta$ -carotene at all four levels between 1.0 and 4.0  $\mu\text{g.}$  at 21 days. However, as the experimental period was extended longer, the growth at the highest level was no longer proportional to the log-dose. The dosage of 4.0  $\mu\text{g.}$  daily is probably too high to be used routinely in assay studies since it borders on the critical level over the period of assay.

The calculated potencies of cryptoxanthin in Ser. I are quite irregular viz., 55.2, 46.4, and 76.0 in the groups given 1.75, 2.75, or 3.75  $\mu\text{g.}$  of this carotenol daily, respectively.

In Ser. II the growth responses on the 19th day gave identical calculated potencies at all three test levels. When compared with the provitamin A activity of  $\beta$ -carotene, cryptoxanthin was found to have a biological activity of 53.1, 56.0, and 54.5% respectively in the three test groups; this gives an average value of 55%. The data show somewhat more variation when calculated on the basis of the 21-day gains. These values were 42.7, 60.4, and 60.5% of  $\beta$ -carotene for the three levels of cryptoxanthin, respectively, with an average potency gain of 55%.

The positive controls in Ser. I gained on the average 242 g., and in Ser. II, 212 g. by the 21st day. This is a greater increase than that shown by any group receiving carotenoids. On the other hand, 94% of the chicks in Ser. I and 61% of the birds in Ser. II in the negative control groups had died before the 21st day. This gives ample evidence that the basal diet is low in vitamin A.

The results obtained confirm the earlier report of Johnson, Swick and Baumann (4) that cryptoxanthin has a lower growth-promoting potency in the chick than  $\beta$ -carotene. The slightly higher potencies for cryptoxanthin reported by these investigators may be due to the fact that the carotenoids were fed at somewhat higher doses than in the present tests. Furthermore, our results fail to confirm With's statement (5) that cryptoxanthin is more potent in the chick than is  $\beta$ -carotene. We find that the ratio, potency of cryptoxanthin/potency of  $\beta$ -carotene, is practically identical in the chick and in the rat (1,2).

#### SUMMARY

Provitamin A potency in the chick of all-*trans*-cryptoxanthin has been found to be 55% of the potency of all-*trans*- $\beta$ -carotene.

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# The Metabolism of Compounds Related to the Carcinogen *N,N*-Dimethyl-*p*-Aminoazobenzene by Rat Liver Slices<sup>1</sup>

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## INTRODUCTION

Rat liver slices have been found to destroy *N,N*-dimethyl-*p*-aminoazobenzene (DMB) and related compounds (1,2,3). This ability of hepatic cells to destroy DMB *in vitro* has been shown to be altered by diets (2,3) which alter the incidence of hepatic tumors produced by DMB (4,5). Liver tumors produced by feeding 3'-methyl-DMB do not destroy appreciable amounts of DMB (6).

In the present study the ability of hepatic cells to destroy 15 related compounds of varying carcinogenic activity has been determined. The extent of conversion of four of the tertiary amino compounds to primary and secondary amines and hydroxy derivatives has also been measured.

## METHODS

The destruction of DMB and related compounds, except the 4'-hydroxy and 4'-sulfonic acid derivatives, was measured as described in our earlier report (2) and depended on the recovery and spectrophotometric estimation of the dye that was not destroyed. One hundred micrograms of the dye was incubated with 150 mg. of liver slices for 60 min. in all experiments. Under the conditions of these experiments the destruction of the dyes was proportional to the period of incubation. The recovery of all of the tertiary amino compounds at zero time was good, 92-98%. However, with all of the primary and secondary amino compounds there was a loss of 30-45  $\mu$ g., even though the tissue was killed by alkali, acid, or heat before the addition of the dye. This loss was prevented by the addition of the reducing agents, thioglycerol or thioglycolic acid, or by keeping the tissue cold throughout homogenization, alkalization,

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and benzene extraction. In determining the rate of destruction of the primary and secondary amino compounds, 5 mg. of thioglycerol was added in each case to prevent this "zero time" loss.

An investigation of this "zero time" loss using the secondary amino compound, *N*-methyl-*p*-aminoazobenzene, revealed that this loss actually represented a destruction of the dye during the period the homogenized tissue was exposed to strong alkali (1.6 *N* KOH) prior to the benzene extraction. The destruction was stopped by the addition of benzene. Extraction with benzene at various time intervals after the addition of the alkali has shown that the amount destroyed was directly proportional to time for 10 min. and then fell off gradually when allowed to stand at room temperature. The amount destroyed in 10 min. was directly proportional to the amount of dye added (25–200  $\mu$ g.) and was proportional to the amount of tissue used, up to 100 mg.; it was independent of further increases in the amount of tissue up to 500 mg., but decreased when still larger amounts of tissue were used. As is shown in Table I, the material responsible for this loss was contained in the heat-coagulated fraction of liver homogenate.

TABLE I  
"Zero Time" Loss of *N*-Methyl-*p*-aminoazobenzene  
(100 mg. liver, 100  $\mu$ g. dye)

	Micrograms dye not recovered
Homogenate	33
Heated homogenate	34
Coagulum	34
Supernatant	0

The amount of the 4'-hydroxy-DMB not destroyed was determined by both the BaCl<sub>2</sub>-acetone-benzene extraction and the direct trichloroacetic acid methods of Mueller and Miller (7). The 4'-sulfonic acid derivative (methyl orange) was determined using the trichloroacetic acid method.

In the experiments on the determination of the amounts of DMB, 2'-methyl-DMB, 3'-methyl-DMB, and 4'-methyl-DMB, which were converted to the *N*-methyl and hydroxy derivatives, the dye mixtures were extracted by the BaCl<sub>2</sub>-acetone-benzene procedure. The acetone-benzene extract was evaporated to dryness under reduced pressure and dissolved in small amounts of benzene. The dyes were then chromatographically separated by using layered columns (8) of mixtures of increasing ratios of alumina to calcium carbonate (increasing adsorbent activity) in 0.5 cm. diameter glass tubing plugged at one end with glass wool. The chromatogram was developed with 10 ml. of a 2:1 benzene-petroleum ether mixture; the column was broken into segments containing the separated dyes and the dyes extracted with benzene, except that the hydroxy derivatives were extracted with methanol. The solvents were evaporated under reduced pressure and the dyes dissolved in 7 *N* HCl and measured spectrophotometrically. The hydroxy derivatives listed in Table III represent total hydroxy derivative calculated as the 4'-hydroxy derivative of the parent compound. It has been shown that the hydroxy derivative of DMB metabolism in fortified liver homogenates is chiefly 4'-hydroxy-DMB (7).

The light-absorption data were obtained with a model DU Beckman spectrophotometer with a slit width of approximately 0.05 mm., and corex cell 1 cm. wide.

The solubility data were obtained by adding 5 mg. of the dyes to 20 ml. of Krebs-Ringer phosphate solution, pH 7.4, and shaking in a water bath at 38°C. for several hours or until equilibrium was reached. The saturated solution was freed of suspended dye by passing through a fine sintered-glass filter, and a suitable aliquot was made up with HCl so that the final acid concentration was 7 *N*. The amount dissolved was determined spectrophotometrically.

## RESULTS

The data obtained on the rate of destruction of the azo dyes are summarized in Table II. The data on destruction of the dyes were obtained by comparing four compounds at the same time, using slices from the same liver. The destruction of all compounds was measured at least five times and the average values are listed in Table II. The *N*-methyl, *N,N*-dimethyl and the *N*-ethyl derivatives of *p*-aminoazobenzene were destroyed by surviving liver cells at approximately the same rate. The *N,N*-diethyl and *N,N*-diethanol were destroyed much more slowly. Methylation of any of the ring positions decreased destruction of the azo compound. In the case of the *N,N*-dimethyl series the rate of

TABLE II  
*Destruction of Compounds Related to p-Aminoazobenzene by Liver Slices*

Compound	Rate of destruction by liver slices <sup>a</sup>	Absorption maximum <sup>b</sup>	Optical density $5 \times 10^{-6}$ <i>M</i> <sup>c</sup>	Solubility pH 7.4 38°C. <sup>d</sup>	Carcinogenic activity <sup>e</sup>
<i>p</i> -Aminoazobenzene	67	500	0.144	mg./ml. 49.	0
<i>N</i> -Methyl- <i>p</i> -aminoazobenzene	65	508	0.245	4.6	1
<i>N</i> -Ethyl- <i>p</i> -aminoazobenzene	63	510	0.212	1.3	0
<i>N,N</i> -Dimethyl- <i>p</i> -aminoazobenzene	60	518	0.228	0.4	1
4'-Methyl- <i>N</i> -methyl- <i>p</i> -aminoazobenzene	50	520	0.217	0.9	0.1
<i>N</i> -Methyl- <i>N</i> -ethyl- <i>p</i> -aminoazobenzene	44	516	0.175	0.4	0.9
3'-Methyl- <i>N</i> -methyl- <i>p</i> -aminoazobenzene	45	510	0.275	0.7	1.8
2-Methyl- <i>N,N</i> -dimethyl- <i>p</i> -aminoazobenzene	44	513	0.224	0.4	0
3'-Methyl- <i>N,N</i> -dimethyl- <i>p</i> -aminoazobenzene	35	520	0.227	0.1	1.8
2'-Methyl- <i>N</i> -methyl- <i>p</i> -aminoazobenzene	35	500	0.119	1.1	0.3
2-Methyl- <i>N,N</i> -dimethyl- <i>p</i> -aminoazobenzene	25	515	0.093	0.2	0.5
<i>N,N</i> -Diethyl- <i>p</i> -aminoazobenzene	25	520	0.089	0.1	0
4'-Methyl- <i>N,N</i> -dimethyl- <i>p</i> -aminoazobenzene	22	530	0.198	0.1	0.1
<i>N,N</i> -Diethanol- <i>p</i> -aminoazobenzene	19	528	0.203	21.	0
4'-Hydroxy- <i>N,N</i> -dimethyl- <i>p</i> -aminoazobenzene	5	465	0.300	0.4	0
4'-Sulfonic acid- <i>N,N</i> -dimethyl- <i>p</i> -aminoazobenzene	3	—	—	—	0

<sup>a</sup> Micrograms destroyed in 60 min. by 150-mg. liver slices.

<sup>b</sup> Determined in 7 *N* HCl. Wavelengths in  $\mu$ .

<sup>c</sup> Azo dyes,  $5 \times 10^{-6}$  *M* in 7 *N* HCl.

<sup>d</sup> Determined in Krebs-Ringer phosphate solution.

<sup>e</sup> Approximate activity (9,10,11,12), DMB reference compound = 1.

destruction was decreased progressively as the methyl group was placed at the 2, 3', 2', and 4' positions. In the case of the *N*-methyl series order of destruction was 4', > 3', > 2'. The placement of a hydroxy or a sulfonic acid group in the 4' position of DMB almost completely prevents destruction. Young (13) has also observed that liver slices do not destroy appreciable quantities of 4'-hydroxy-DMB. However, the 4'-hydroxy compound was not found to inhibit the destruction of DMB when both were incubated with the slices at the same time.

TABLE III  
*The Metabolism of Azo Dyes by Liver Slices*

Compound 100 mg.	Amount destroyed	Compounds recovered					
		$\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{R}-\text{N} \\ \diagdown \\ \text{H} \end{array}$		$\begin{array}{c} \text{H} \\ \diagup \\ \text{R}-\text{N} \\ \diagdown \\ \text{H} \end{array}$		$\begin{array}{c} \text{CH}_3 \\ \diagup \\ \text{HO}-\text{R}-\text{N} \\ \diagdown \\ \text{CH}_3 \end{array}$	
	$\mu\text{g.}$	$\mu\text{g.}$	% <sup>b</sup>	$\mu\text{g.}$	%	$\mu\text{g.}$	%
DMB	75	1.3	1.7	0	0	3.7	4.9
	76	0.7	0.92	0	0	3.2	4.2
	76	0.75	0.99	0	0	3.5	4.6
3'-Methyl-DMB	41	1.9	4.6	0	0	1.1	2.7
	56	1.3	2.3	0	0	1.2	2.1
	48	1.2	2.5	0	0	1.2	2.5
2'-Methyl-DMB	20	1.2	6.0	0	0	ca. 1	
	30	4.0	13.3	0	0	ca. 1	
4'-Methyl-DMB	20	1.4	7.0	0	0	0	0
DMB (rice-diet rats)	42	1.0	2.4	0	0	1.7	1.7
	49	0.8	1.6	0	0	1.8	2.7
	28	2.4	8.6	0	0	1.5	5.3
	22	1.0	4.5	0	0	0.7	3.1

<sup>a</sup> Total hydroxy dyes calculated as the 4'-OH-R-N(CH<sub>3</sub>)<sub>2</sub> derivative.

<sup>b</sup> Per cent of dye destroyed.

It is evident from the data in Table II that there is no simple correlation between the rate of dye destruction and the solubility of the dye in the solution used in these experiments, namely Krebs-Ringer phosphate pH 7.4. The data in Table II also indicate that there is no appar-

ent relationship between the rate of destruction of these compounds by liver tissue *in vitro* and their carcinogenic activity *in vivo*. The absorption maxima of the various compounds are essentially the same as those reported for most of these compounds in an aqueous 7 *N* HCl-ethanol mixture (14).

The data obtained on the conversion of DMB and of certain related compounds to their demethylated and hydroxylated derivatives are shown in Table III. In the case of all four compounds studied, no primary amino derivative was detected. However, in each case significant quantities of the *N*-methyl derivatives accumulated. Hydroxy

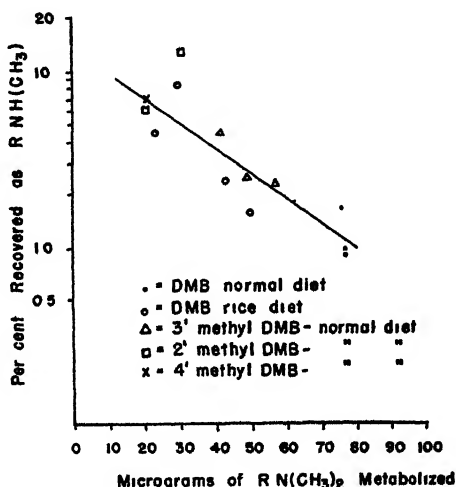


FIG. 1. Accumulation of  $R-NH(CH_3)$  as a function of  $R-N(CH_3)_2$  destroyed by liver slices.

derivatives were recovered in each case with the exception of the 4'-methyl-DMB. The accumulation of the *N*-methyl derivatives was greater in the case of the 2', 3' and 4' methyl derivatives of DMB than for DMB itself. These data on the accumulation of an *N*-methyl derivative as a function of the amount of the tertiary parent compound destroyed are plotted in Fig. 1. It can be seen that as the amount of the tertiary compound destroyed is reduced by the addition of methyl groups on the prime ring of DMB, the per cent of the azo dye recovered as the *N*-methyl derivative increased. As it has been found earlier that the maintenance of rats on a brown rice ration for periods greater than

one week consistently reduced the ability of their livers to metabolize DMB *in vitro*, 4 rats maintained on this ration for from 18 to 22 days have been examined. As is evidenced in Table III, both *N*-methyl and hydroxy derivatives were recovered. A proportionately higher amount of the *N*-methyl compound was obtained with rice-diet rats than with the normal-diet rats when expressed as a percentage of DMB destroyed. These animals are included in the data plotted in Fig. 1.

In contrast to the increased percentage of the *N*-methyl derivative recovered when the azo dye examined was less readily destroyed than DMB, or when the ability of rat liver to destroy DMB was reduced by a deficient diet, the percentage of dye destroyed that was converted to a hydroxy derivative was either unchanged or decreased.

### DISCUSSION

The finding that DMB, 2'-methyl-DMB, and 3'-methyl-DMB are metabolized *in vitro* to form small amounts of hydroxy derivatives indicates that direct hydroxylation without cleavage of the azo linkage represents a pathway of metabolism in intact liver cells as well as in fortified liver homogenates (6) and *in vivo* (12). The observation that 4'-hydroxy-DMB is not appreciably destroyed by liver slices indicates that this is not a major pathway, as only 5% or less of the DMB destroyed by liver slices can be accounted for as hydroxy azo dyes. That 4'-hydroxy-DMB is not readily destroyed by fortified liver homogenates has been reported by Young (13) who also has reported that these homogenates form more secondary amino and hydroxy derivatives from 3'-methyl-DMB than from DMB.

The observation that a greater percentage of the DMB that is metabolized is converted to the *N*-methyl derivative if the amount of azo compound destroyed by rat liver is decreased by deficient diets, or by methylation of the prime ring, indicates that the demethylation of the tertiary compounds is not a limiting reaction in either case. In contrast, the production of hydroxy derivatives of DMB and related compounds appears to decrease more than the over-all dye destruction under similar conditions.

That demethylation of DMB and related tertiary compounds is necessary under certain conditions for the destruction of these azo dyes is indicated by the observation that only secondary and primary amino azobenzenes are destroyed when they are exposed to low concentrations of homogenized liver in the presence of alkali. This latter type of de-

struction differs from that obtained in fortified homogenates and liver slices in that it is not prevented by heat coagulation, and the reaction occurs only in the presence of strong alkali. Moreover, it is blocked by reducing agents, such as thioglycerol, which in equivalent concentrations are without effect on destruction in liver slices or fortified homogenates. This observation suggests that the loss is due to oxidation, but hydrogen peroxide oxidizes the tertiary compounds as well as the primary and secondary ones. Autoxidizing linoleic acid (15) destroys all three groups of compounds. The alkali-catalyzed destruction of the primary and secondary amino azo compounds by heat-coagulated liver homogenate was not affected by diets which had been shown earlier to influence DMB destruction (2,3).

The results of the study on the rates of destruction of the various azo dyes by liver slices do not indicate that there is a correlation between the rate of dye destruction or the production of *N*-methyl or hydroxy derivatives and carcinogenic activity.

#### ACKNOWLEDGMENTS

We are greatly indebted to Dr. M. L. Crossley for the azo compounds used in these experiments, and to Mrs. Edith Shapiro and Miss Elizabeth Hurd for technical assistance.

#### SUMMARY

1. Liver slices destroy *N*-ethyl but not *N*-methyl derivatives of *p*-aminoazobenzene more slowly than *p*-aminoazobenzene. Alkylation of the 2, 2', 3', and 4' positions also decreases the rate of destruction. The 4'-hydroxy or 4'-sulfonic acid derivatives of *N,N*-dimethyl-*p*-aminoazobenzene are not destroyed at an appreciable rate under similar conditions.

2. *p*-Aminoazobenzene and secondary amino, but not tertiary amino derivatives, are destroyed when they are exposed to small concentrations of liver homogenates in the presence of strong alkali. This loss can be prevented by reducing agents such as thioglycerol, or by keeping the temperature at 0°C.

3. *N*-Methyl and hydroxy derivatives are formed from *N,N*-dimethyl-*p*-aminoazobenzene, 2'-methyl-*N,N*-dimethyl-*p*-aminoazobenzene and 3'-methyl-*N,N*-dimethyl-*p*-aminoazobenzene when they are metabolized by liver slices.

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# The Isolation of Isoquercitrin From Air-Dried Tobacco

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## INTRODUCTION

Two flavonoid pigments have been isolated from tobacco by previous workers. Hasegawa (1), and Neuberg and Kobel (2) obtained rutin (quercetin-3-rhamnoglucoside) from fresh tobacco leaves. Later, Couch and Krewson (3) isolated rutin from high grade green and flue-cured tobacco. Kourilo (4) obtained isoquercitrin (quercetin-3-glucoside) from the leaves of unfermented "Tyk-koulak" tobacco.

Previous to the work described here, however, no report has been found of the actual isolation of a flavonoid pigment from air-cured, low grade tobacco. Air-curing, according to Couch and Krewson (3), causes the rutin to be lost. Neuberg and Kobel (5) reported that rutin is destroyed enzymatically in the process of air-curing. No specific mention of the status of isoquercitrin after air-curing has been located. In the investigation reported here, the authors were interested in checking for the possible presence of small remaining quantities of isoquercitrin or other flavonoid pigments in low grade tobacco after air-curing. It has been found that the method of Kourilo (4) is not sensitive enough for minute quantities of isoquercitrin, for no flavonoid pigment could be isolated by that method from air-dried tobacco. This paper describes another method whereby a flavonoid pigment has been isolated from air-dried tobacco in minute quantities. By the use of paper partition chromatography, as well as classical methods, this pigment has been identified as isoquercitrin.

## EXPERIMENTAL

### *Isolation of the Glycoside*

Low grade lug Burley tobacco, *Nicotiana tabacum*, grown and air-dried on the farm of the University of Kentucky Agricultural Experiment Station in 1942, was finely divided in a hammer mill and stored in heavy cloth bags until used in 1947 and 1948.

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Two hundred g. of this tobacco was extracted with 1.5 l. of boiling water for 1.5 hr. and filtered through glass wool. The residue was washed with hot water until the washings had only a faint yellow color.

The extract was then concentrated *in vacuo* to 450 ml., filtered again while hot, and the residue washed with hot water. After cooling the filtrate and washings, about 2 l. of acetone was slowly added with vigorous stirring. After 1.5 hr. the resulting residue was thoroughly washed and extracted with acetone. These acetone washings were combined with the acetone-water filtrate. Asparagine crystals were obtained from this filtrate on standing (6). The acetone-water solution was decanted from the asparagine, the acetone removed by distillation, and the remaining water solution filtered.

Addition of normal lead acetate solution at pH 4.5 gave a light yellow precipitate which tested negatively for flavonoid pigments. The filtered water solution was next treated with 200 ml. of basic lead acetate solution. The yellowish precipitate was suspended in 150 ml. of water, and 200 ml. of ethyl alcohol containing 10 ml. of concentrated sulfuric acid was added. After removal of lead sulfate, the solution was buffered with sodium acetate and then concentrated under reduced pressure to about 75 ml. The dark tarry mass which formed in the solution after standing 2 days was separated and dissolved in alcohol. After concentrating this solution to 5–10 ml., the viscous liquid was poured into 200 ml. of ether. A black precipitate which formed was discarded. The ether was evaporated leaving a small volume of reddish brown solution. A small amount of dark solid which appeared on cooling was discarded. After standing in the cold for 1 week a small amount of a bright yellow precipitate formed on the sides and bottom of the flask. This precipitate was treated with successive small portions of ether (contaminated with the usual amount of alcohol) until the extracts were practically colorless. The combined ether extracts were concentrated to about 5 ml. Hot water was added to the point of incipient precipitation. Cooling produced a small quantity of light yellow powder which was recrystallized several times by dissolving in about 1 ml. of alcohol and precipitating with water. A little over 5 mg. was obtained after drying—a yield of approximately 0.0025%.<sup>2</sup>

### *Purification and Identification of the Isoquercitrin*

The technic of paper partition chromatography (7), as applied to flavonoid pigments (8), was helpful in the purification and identification of the isolated pigment. Two zones were produced when the tobacco flavonoid was chromatographed on Whatman No. 1 paper in the various irrigating solvents. The larger of these two zones corresponded in  $R_f$  values with authentic isoquercitrin. The smaller zone corresponded with the  $R_f$  values of quercetin. The  $R_f$  values for the isoquercitrin and quercetin zones, respectively, in the various solvents were as follows: *n*-butanol-acetic acid-water (40:10:50%/vol., resp.): 0.73, 0.81; *o,p*-cresols-water: 0.29–0.30, 0.43; phenol-water: 0.53, 0.39. Authentic isoquercitrin and quercetin when chromatographed on control strips in the same chamber with the tobacco flavonoid exhibited  $R_f$  values in agreement with the respective values quoted above.

The fluorescence of the separated pigment zones in ultraviolet light after spraying with various chromogenic sprays (8) was also used in determining the identity of the

<sup>2</sup> The yield of 0.0025% represents a minimum figure. Considerable losses may have occurred during the isolation procedure due to hydrolysis and oxidation.

two pigment zones. The main zone exhibited fluorescent colors identical with those produced by authentic isoquercitrin when sprayed with the various reagents. The smaller zone corresponded in color tests with authentic quercetin.

The principle pigment zone—that of purified isoquercitrin—was cut out of three filter paper strips, after paper partition chromatography in butanol-acetic acid-water, and the pigment extracted with 95% ethyl alcohol. The ultraviolet absorption spectrum of the flavonoid solution was then obtained in the Beckman Model DU spectrophotometer. Three untreated control strips were chromatographed in the same solvent system and a section of each strip corresponding in area and location with the isoquercitrin zone was removed and extracted with ethyl alcohol in order to provide a solvent blank for the instrument. Absorption maxima of the purified pigment were observed at  $258\text{ m}\mu$  and  $360\text{--}1\text{ m}\mu$ . Minima were observed at  $238\text{--}9\text{ m}\mu$  and  $285\text{ m}\mu$  (Fig. 1). The plotted curve corresponded in shape with the absorption spectrum of authentic isoquercitrin obtained in this laboratory.

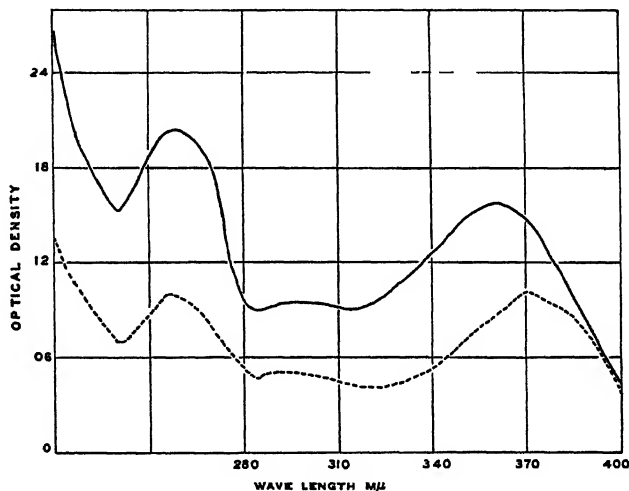


Fig. 1. ----- Spectrum of impure isoquercitrin, obtained from air-dried tobacco, prior to paper partition chromatography. ——— Spectrum of isoquercitrin, from air-dried tobacco, after purification by paper partition chromatography.

A technic similar to that above was used in obtaining the absorption spectrum of the aglycone formed by hydrolysis of the isoquercitrin. Maxima of the aglycone were identical with those of authentic quercetin (from rutin).

The method of Partridge (9) was followed in identifying the sugar obtained after hydrolysis of the isoquercitrin. Only one sugar was observed on the developed paper chromatogram strip after spraying with ammoniacal silver nitrate solution and heating. The  $R_f$  value, 0.19–0.21, in butanol-acetic acid-water (40:10:50%/vol., resp.) corresponded well with the value obtained for known glucose, 0.18–0.20.

Identification of the glucose portion of the isoquercitrin was also made by the preparation of the osazone, using the procedure described in Bodansky and Fay (10) with 0.01 the amounts prescribed. A 1 mg. sample of glucose was used to form a glucosazone for comparison. Only the glucosazone was observed in the test sample.

## DISCUSSION

The small yield of isoquercitrin obtained indicates that its presence in this tobacco is probably quite low. While the isoquercitrin content of Kentucky Burley tobacco prior to air-curing has not been reported, it is believed that air-curing tends to destroy isoquercitrin as well as rutin.

It cannot be stated with certainty that all previous reports on the isolation of flavonoid pigments from tobacco have been the result of starting with either fresh plants or with flue-cured plants, due to the inadequate descriptions of the tobaccos used in the investigations. Indications are strong that the analyses were made on fresh or flue-cured tobaccos. Thus, it is probable that this work is the first isolation of a flavone glycoside from air-cured tobacco and perhaps from tobacco of such low grade.

This is also the first report of the isolation of isoquercitrin from Kentucky Burley tobacco.

The spectrum of the tobacco flavonoid obtained prior to paper partition chromatography has been included in Fig. 1. The change in the ultraviolet absorption spectrum of the isoquercitrin following purification by paper chromatography demonstrates the advantage of this method in the purification and identification of flavonoid pigments. The shift in the absorption maximum from 370 to 361  $m\mu$  is probably due to the removal of quercetin (maximum at 375  $m\mu$ ) from the sample.

The butanol-acetic acid-water mixture used in chromatography undergoes esterification on standing. As increasing amounts of butyl acetate are formed, the water content of the butanol layer is diminished. A shift in  $R_f$  values results. Slight differences in  $R_f$  values have also been noted in the phenol-water system. The variation in the latter instance is thought to be due to differences in purity of various lots of phenol. For identification purposes it has been found expedient to run known samples of a flavonoid alongside the unknown pigment in the same chamber and solvent system in order to avoid errors due to a shift in  $R_f$  value.

## ACKNOWLEDGMENTS

The pure isoquercitrin was kindly furnished by the Pharmacology Laboratory, Bureau of Agricultural and Industrial Chemistry, Albany, California. The Kentucky Burley tobacco was generously donated by the Agronomy Dept., Univ. of Kentucky Agr. Exptl. Sta., Lexington, Ky., and by Dr. R. N. Jeffrey.

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## SUMMARY

1. Isoquercitrin has been isolated in small yields from air-dried, low grade Kentucky lug Burley tobacco, *Nicotiana tabacum*.
2. The details of the isolation and identification have been given.
3. This is probably the first reported isolation of a flavone glycoside from tobacco after air-drying and long storage, and perhaps, from tobacco of such a low grade.

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## The Biogenesis of the Anthocyanins. II. Evidence for the Mediation of Copper in Anthocyanin Synthesis <sup>1</sup>

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### INTRODUCTION

In a previous paper (5), some of the general conditions governing anthocyanin formation in *Spirodela oligorrhiza* Kurtz were reported. It was shown, *inter alia*, that pigment formation was affected by the concentration of inorganic micronutrients in the medium. Some of these (B, Mo, Zn) when present in minimal amounts caused an increase in anthocyanin in the plant tissues; this was considered to be a non-specific response, due to the sharp decrease in growth under starvation conditions. An increase in the copper concentration of the medium to a toxic level likewise resulted in increased pigment, again probably as a concomitant of decreased growth. In all these cases the growth was reduced to about the same level and the pigment concentration increased to a comparable degree; and the general relation implied above between growth rate and pigment concentration was demonstrated to hold consistently.

Attempts to study the effects of copper starvation failed, however, because of the contamination of other components of the medium with quantities of copper sufficient to support normal growth and metabolism. Even when iron tartrate, one of the major sources of copper contamination, was made up from purified tartaric acid and iron reduced in hydrogen, this remained true. The pigments under investigation are of a polyphenol nature; and some of our experiments (see Ref. 1) have

<sup>1</sup> Part of this work was supported by a grant from the Rockefeller Foundation, to whom we wish to express our thanks. The authors' thanks are also due to Miss Babette Solomon for carrying out the experiments of Table V.

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indicated a close relation between anthocyanin production and the metabolism of such substances as catechol and tyrosine. In particular, the addition of small amounts of catechol to the medium resulted in its conversion to a black precipitate and in a marked inhibition of anthocyanin formation. These experiments will be described in detail later. Because of the known importance of copper enzymes in polyphenol systems, the role of copper was felt to be worth more careful study. For this purpose the copper concentration in the medium for *Spirodela* was altered by indirect methods, and the results described herein give clear evidence of the participation of copper in the formation of anthocyanin.

The experimental use of so-called "copper poisons," *i.e.*, reagents which form chelate complexes with copper, is well known with both plant and animal material. The chemistry of many of the reactions has not been worked out in detail, and the reagents have shown varying degrees of effectiveness, depending upon the compound used and upon the particular experimental conditions employed. Three of the best known copper-combining reagents have been used in this study: phenylthiocarbamide, salicylaldoxime and the sodium salt of diethyldithiocarbamate. Of these, the first-named has given the clearest results.

## METHODS

The methods used were in large part those reported previously (5). Plants were grown in sterile culture on a modified Hoagland's solution (Medium I), usually both with and without the addition of  $2.5 \times 10^{-2} M$  sucrose; other compounds were added to the medium before autoclaving. Plants were assayed at 2 weeks of age unless otherwise noted, and the absorption of the acidified pigment solutions was determined in a Klett-Summerson photoelectric colorimeter with a Klett No. 54 filter.

In one experiment nongrowing plants were used. The technique followed here was that plants grown under low light intensity were transferred in uniform, fairly large quantities to flasks containing nonnutrient solutions; these were then placed under high illumination in the light-room, in this case for a period of 4 days, and finally assayed as above. The pigment content of aliquots of the inoculum was also assayed, and data are presented as the increment in pigment units/g. fresh weight, after exposure for 4 days.

Reducing sugars were determined with the reagent of Somogyi (4), using iodometric titration rather than colorimetry. Sucrose was measured, after 24 hours incubation with invertase, by the same method. Data for both sugars and pigment concentration are referred to the fresh weight of the plants.

## EXPERIMENTAL

*Action of Phenylthiocarbamide (PTC) on Growth and Pigment Formation*

Table I shows the effect upon growth and pigment formation in *Spirodela* of the addition to the nutrient medium of various concentrations of phenylthiocarbamide (PTC). It may be seen that the complete range is a narrow one, from little or no effect at  $10^{-5}$  M to an almost complete inhibition of growth at  $3 \times 10^{-4}$  M; the few new fronds which appeared at the higher concentration were tiny and malformed, while the older fronds were clearly dying. Either there was not enough copper to support growth, or the PTC itself had become toxic at this concentration.

TABLE I

*Effect of Phenylthiocarbamide on Growth and Pigment Formation in Spirodela oligorrhiza*  
Age of plants: 2 weeks.

Medium	Growth		Pigment	
	g /culture	% of control	units/g.	% of control
I <sup>a</sup> (inorganic)	0.29	100	369	100
+PTC $10^{-5}$ M	0.27	93	363	98
+PTC $3 \times 10^{-5}$ M	0.38	131	236	64
+PTC $10^{-4}$ M	0.30	103	106	29
+PTC $3 \times 10^{-4}$ M	0.05	17	—	—
I + sucrose 0.025 M	0.28	100	640	100
+PTC $10^{-5}$ M	0.29	104	537	84
+PTC $3 \times 10^{-5}$ M	0.29	104	352	55
+PTC $10^{-4}$ M	0.27	96	163	25
+PTC $3 \times 10^{-4}$ M	0.07	25	—	—

<sup>a</sup> For composition of Medium I, see Ref. 5.

With PTC at a concentration of  $10^{-4}$  M, however, growth was essentially normal, while the pigment concentration was reduced to 25–30% of that of the controls. This effect was seen both in the presence and absence of sucrose, the absolute pigment concentrations in the sucrose medium being always higher than in the corresponding inorganic medium. A concentration of  $10^{-4}$  M PTC was therefore adopted for

further work. In this and in subsequent tables it may be seen that PTC always reduces the anthocyanin to less than half its level in the controls. The removal of copper by PTC also modifies the dependence of anthocyanin formation upon the sugar content of the medium; in all experiments the addition of  $10^{-4}$  M PTC to sucrose medium brought the pigment concentration not only below that of the sucrose control but below that produced on Medium I alone.

### *Mechanism of the Action of PTC*

Since the previous experiments had indicated a close relationship between anthocyanin synthesis and the sugar supply, it was thought possible that the decrease in pigment caused by PTC might be due to a decrease in sugar content. Accordingly, the influence of PTC on the

TABLE II  
*Sugar Content of S. oligorrhiza in Relation to Phenylthiocarbamide Treatment*  
Age of plants: 2 weeks.

Medium	Pigment	Reducing sugars as glucose	Sucrose as glucose
	<i>units/g.</i>	<i>mg./g.</i>	<i>mg./g.</i>
I	240	0.526	0.987
I+PTC, $10^{-4}$ M	113	0.555	1.24
I+sucrose, 0.025 M	410	0.615	1.05
I+sucrose+PTC, $10^{-4}$ M	181	0.775	3.08

sugar content of *Spirodela* fronds was examined. Plants grown with and without sucrose, in the presence and absence of PTC, were subjected to analysis for reducing sugars and for sucrose. The results are shown in Table II. While reducing sugars accumulated somewhat over their level in the controls in both media when grown with PTC, the effect upon sucrose was far more striking. Apparently sucrose is not normally accumulated during the two-week period, even when supplied in the medium, beyond about 1 mg./g. fresh weight; in other experiments it was found that even after 8 weeks growth on sucrose medium, while the reducing sugar content had mounted considerably, the sucrose concentration within the plants was only about 1.28 mg./g. But in the presence of PTC an increase of 25% in sucrose content appeared after two weeks on Medium I, and when sucrose was fed in addition, its

concentration in the tissue rose to some 300% of the control value. It follows from these data that the possibility mentioned above is excluded; the effect of PTC in lowering the pigment content is not achieved by lowering the sugar content of the plant tissues. Instead, the PTC may operate on pigment formation through interference with sugar metabolism.

Since the dry weight of *Spirodela* is about 12% of its fresh weight, 1 g. of plant material contains about 0.88 g. of water. Thus it may be calculated that the average sucrose concentration in the plant liquids is about  $3.3 \times 10^{-1} M$  in normal photosynthetic plants, whether or not they are grown on sucrose; but in the plants grown on Medium I plus PTC this is raised to  $4.1 \times 10^{-3} M$ , and in the plants grown on sucrose plus PTC the concentration reaches  $10 \times 10^{-3} M$ . This still

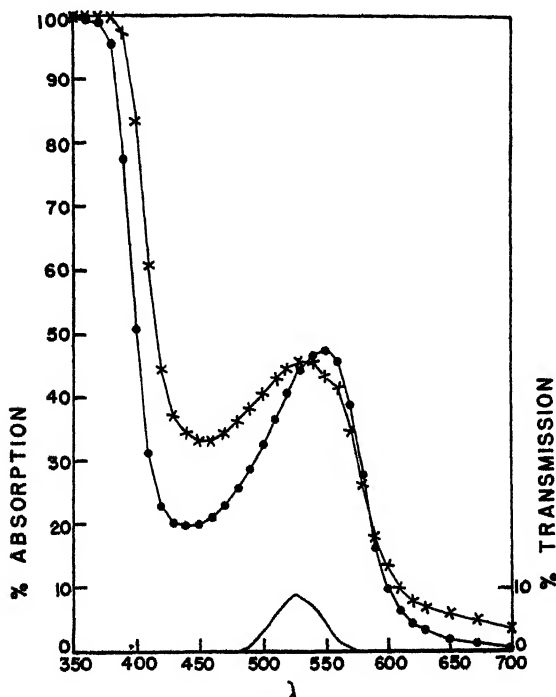


FIG. 1. Absorption spectra of *Spirodela* anthocyanins. Circles, normal pigment; crosses, pigment formed in presence of PTC. The transmission of the filter used is shown at the bottom.

does not, of course, represent accumulation of sucrose from the medium against a concentration gradient, since the medium itself is  $25 \times 10^{-3} M$ .

The effect of PTC on the pigment of *Spirodela* is qualitative as well as inhibitory. Acid extracts from plants grown on PTC are orange red to the eye rather than the rose red of those from normal plants; absorption spectra for both are given in Fig. 1. One practical result of this difference is to make the pigment data given, derived from readings obtained with the Klett No. 54 filter (peak transmission 525  $m\mu$ ), increase the apparent relative pigment of the PTC plants; thus all figures on PTC inhibition based on such data are in fact underestimated. The meaning of this change in the absorption spectrum is as yet obscure. Enough PTC pigment has not been collected to ascertain whether the change represents an alteration in the basic structure of the pigment molecule, or an admixture with some colored intermediate product which has been allowed to accumulate.

TABLE III

*Pigment Formed During 4-Day Exposure by Plants Floated on Nonnutrient Solutions with and without Phenylthiocarbamide*

Solution	Pigment units/g		Pigment in PTC as % of control
	No PTC	PTC $10^{-4} M$	
Water	394	142	36
Sucrose, 0.025 $M$	596	203	34

In the experiments reported above, PTC presumably acts by removing copper from the nutrient solution so that it is no longer available to the growing plants. In order to find out whether PTC could actually combine with copper already inside the plant cells, a similar experiment was carried out in the absence of nutrient. The procedure was described previously (p. 80). Results are shown in Table III. It is evident that the relations already elucidated for growing plants hold for nongrowing ones also; PTC inhibits pigment formation to the same degree as before, and pigment formed on sucrose when PTC is present is less than that formed in the absence of both compounds. PTC thus apparently acts by combining with copper already *in situ*, not merely by withholding it from plants during their growth.

*Action of Other Copper-Combining Reagents*

In addition to PTC, two other copper-combining reagents were employed: salicylaldoxime (SAO) and sodium diethyldithiocarbamate (DDC). The results of growing *Spirodela* in organic nutrient medium in presence of all of these compounds are summarized in Fig. 2. The same general effect may be seen at the highest concentration of each reagent, namely, a significant decrease in the number of pigment units formed/g. of plant tissue. It is to be noted that the effective concentrations of the three reagents were different. More important, however, was the fact that no separation between the concentrations of inhibitor effective upon growth and upon pigment formation could be obtained with SAO and DDC; in these cases the concentrations inhibiting pigment formation were identical with those inhibiting growth. Results very similar to those shown in Fig. 2 were also obtained in sucrose medium; in other words the addition of sucrose does not increase the separation between pigmentation and growth. PTC is thus the only one of the three inhibitors providing a useful concentration at which growth is completely normal while pigment is strongly inhibited. PTC is also the only inhibitor so far tried with which pigment inhibition can be shown to vary linearly with the logarithm of the inhibitor concentration, a relation which holds also on sucrose medium (cf. data given in Table I). It is of interest to note here that if these curves were to be extrapolated to the base line, the pigment inhibition curve would intercept at a slightly lower concentration of PTC than that needed to prevent growth completely; this is true both with and without added sucrose. This means in effect that the pigment system is more sensitive to PTC than the growth system, even at its extremity. Experimental verification of these relations would be of great interest in connection with the whole problem of the possible function of anthocyanin pigments, although somewhat difficult to obtain because of the small quantities of material involved.

It was noted that at the highest usable concentration of SAO, namely  $10^{-4}$  M, the small amount of pigment formed had (as did the PTC extracts) an orange rather than a rose hue; but there was not enough for spectrophotometric analysis. The relatively weak effect of DDC may possibly be due to its partial decomposition on being autoclaved. Furthermore, there was a heavy, white precipitate in the medium at the

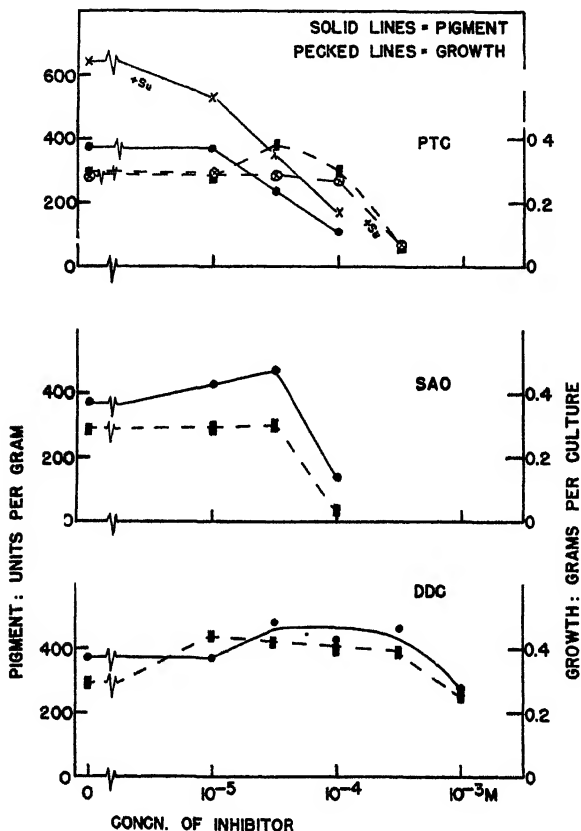


FIG. 2. Growth and anthocyanin formation in the presence of serial concentrations of copper-combining compounds. Circles, growth (right-hand ordinate); squares, anthocyanin (left-hand ordinate). PTC, phenylthiocarbamide; SAO, salicylaldehyde; DDC, diethyldithiocarbamate. All cultures after 2 weeks on Medium I. In PTC figure, crosses (pigment) and crosses in circles (growth) represent experiments in the presence of sucrose (0.025  $M$ ). In the other figures, comparable series in the presence of sucrose gave results similar to those shown, though with higher absolute yields of pigment.

higher concentrations which suggested possible combination with constituents of the medium other than copper; this made the use of still higher concentrations seem inadvisable. Pigment extracts were of normal hue.

*Balance Between the Concentration of PTC and Copper*

To study the relation between the inhibitory effect of PTC and the copper content of the medium, copper was added to media with and without PTC in three concentrations ranging from the standard amount ( $3.3 \times 10^{-8} M$ ) to a clearly toxic one, 100 times higher. After the plants had grown on these media for 2 weeks, they were removed from the light-room, and to two groups of the flasks containing PTC and normal low copper there was added, aseptically, sterile copper sulfate

TABLE IV

*Effect of Copper upon the Inhibition of Anthocyanin Formation by Phenylthiocarbamide*

Copper concentrations represent copper actually added to the medium, in addition to that fortuitously present. Age of plants: 3 weeks.

Medium	Time of copper addition	Growth	Pigment
		<i>g./culture</i>	<i>units/g.</i>
I			
Cu $3.3 \times 10^{-8} M$	At start	0.61	112
Cu $33.0 \times 10^{-8} M$	At start	0.40	112
Cu $330.0 \times 10^{-8} M$	At start	0.17 <sup>a</sup>	477
I+PTC $10^{-4} M$			
Cu $3.3 \times 10^{-8} M$	At start	0.94	54
Cu $33.0 \times 10^{-8} M$	At start	0.97	60
Cu $330.0 \times 10^{-8} M$	At start	0.42	206
Cu $33.0 \times 10^{-8} M$	After 2-week growth	0.75	72
Cu $330.0 \times 10^{-8} M$	After 2-week growth	0.42	108

<sup>a</sup> Plants have typical appearance of high copper growth inhibition: small fronds, short roots.

solution in quantities sufficient to bring their copper content up to that of the more concentrated original media. All plants were returned to the light-room for a final week, and then assayed; results are presented in Table IV. Data are given for Medium I only, but the behavior of the plants was essentially similar with sucrose.

Whether the additional copper was included in the medium from the start or only added for the last week of growth, its effect was qualitatively the same, namely to offset, at least in part, the inhibiting effect of the PTC. In neither case, of course, was pigment formed in presence

of PTC to the same extent as in its absence at a given copper concentration. It should be emphasized here that the inverse relation between growth and pigment in these particular data cannot be considered to be causal for several reasons, most simply because the plants at  $330 \times 10^{-8}$  *M* copper are the only ones whose growth was sufficiently inhibited to have been thus affected. Similar experiments in which manganese instead of copper was added in concentration from  $1.8 \times 10^{-8}$  to  $9 \times 10^{-6}$  *M* showed no effect, except some inhibition at the highest manganese concentrations. Another experiment with a larger range of copper concentrations is given in Table V. In this table the figures have been corrected for the amount of pigment in the initial inoculum, so

TABLE V  
*Experiment as in Table IV*

Age of plants at assay: 2 weeks. Pigment figures corrected for pigment present in the inoculum.

Medium	Copper added $\times 10^{-8}$ <i>M</i>	Growth <i>g</i> / culture	Pigment concentration <i>units/g</i>	Total pigment produced <i>units</i>
I	3.3	0.38	230	87.4
I+PTC $10^{-4}$ <i>M</i>	3.3	0.17	49	8.3
I+PTC $10^{-4}$ <i>M</i>	33	0.37	39	14.4
I+PTC $10^{-4}$ <i>M</i>	100	0.22	48	10.6
I+PTC $10^{-4}$ <i>M</i>	200	0.17	106	18.0
I+PTC $10^{-4}$ <i>M</i>	330	0.13	194	25.2

that the inhibiting effect of PTC on the new formation of pigment is more clearly seen. It is evident that though the addition of copper by no means fully offsets the inhibition due to PTC, it certainly acts in the expected direction. The figures for pigment per culture show that in the high copper concentrations, which have some inhibiting effect on growth, the total amount of pigment formed is increased.

## DISCUSSION

There seems no reasonable doubt that the effect of PTC in inhibiting anthocyanin formation is due to its copper-combining properties. This is supported by the results with salicylaldoxime, since at the pH of the nutrient medium used, this reagent combines with copper alone among the ions present (see Ref. 2, Table 29). Partial reversal of the PTC

inhibition, both by titration with copper in the original medium and by addition of copper after some growth has taken place, supports the same conclusion. Thus the mediation of copper in the mechanism of anthocyanin synthesis must be postulated.

It would of course be desirable to demonstrate this directly in copper-free medium, but unfortunately it appears from other experiments in this laboratory (3) that the development of true copper deficiency by *Spirodela* at pII 5 is extremely slow and that the amount of copper transferred in the inoculum is sufficient for more than 4 weeks growth. Experiments in this direction are, however, under way.

These considerations lead directly to the second question, which is that of the site of PTC activity. In cultures on nutrient medium, the simplest view is that the PTC combines with copper in the medium, making it unavailable for the growing plants; and if this were indeed the mechanism, then the production of identical results with copper-free medium would theoretically be possible. The results with the non-growing plants, however, point in another direction, for here PTC must almost certainly combine with copper already within the plant; and the great similarity noted above between the inhibition of pigment formation in growing and in nongrowing plants makes it probable that this is true with growing cultures as well. In this case the copper content of the medium would be relevant in terms of its effect on the PTC in the external solution, and also on the equilibrium between PTC and copper within the cells; but whether the absence of copper in the external medium could affect its concentration within the plant would depend, given sufficient permeability, on the form in which copper was present in the cells.

Whether the copper acts of itself or as the prosthetic group of an enzyme is, of course, indeterminable from the present evidence. Even if it be assumed that one of the enzymes involved in anthocyanin formation contains copper, it cannot be assumed further that this is identical with the tyrosinase known to exist in *Spirodela* or with polyphenol oxidase. Some evidence bearing on this question will be presented in a later paper. Unfortunately it is not yet clear whether or no there is more than one polyphenol oxidase, nor is it certain that such enzymes play an essential part in the metabolism of normal, unwounded plant tissue. Considerably more work will be required before a definitive step in anthocyanin synthesis can be related to a specific enzyme.

## SUMMARY

1. Following indications that the copper content of the nutrient medium exerts an influence on anthocyanin formation in *Spirodela*, the plants were raised in presence of copper-combining reagents.

2. Phenylthiocarbamide (PTC), in concentrations too low to inhibit growth, reduces anthocyanin formation.

3. This effect is not brought about by lowering the sugar content of the plant tissue, because reducing sugars, and to a greater extent, sucrose, accumulate in the plants grown in the presence of PTC more than in the controls.

4. A similar inhibition of formation of anthocyanin occurs in non-growing plants suspended in PTC solution with or without sucrose; it is concluded that PTC combines with copper actually present in the cells and not merely with external copper ions.

5. Two other copper-combining reagents, salicylaldoxime and diethyldithiocarbamate, also inhibit anthocyanin formation, but their effects on pigmentation are accompanied by inhibition of growth.

6. Changes in the absorption spectrum of the anthocyanin formed in the presence of the copper-combining reagents indicate that there are qualitative differences in the pigments.

7. The addition of copper to the medium, within narrow concentration limits, increased the formation of anthocyanin in the presence of PTC.

8. It is concluded that copper, probably in the form of a copper-containing enzyme, participates in the formation of anthocyanin.

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# Hexadecenoic Acid as a Growth Factor for Lactic Acid Bacteria

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## INTRODUCTION

Unsaturated fatty acids of the 18 carbon atom series, especially oleic acid, have been found by several investigators to be (a) essential growth factors for some lactic acid bacteria in a complete medium which includes biotin (1,4), and (b) growth-stimulating for others but not essential for growth in a variety of media; they are essential for growth only when biotin is absent from the medium (2,3,4,8). Saturated fatty acids have been found inactive as promoters of bacterial growth (3). Effects of saturated fatty acids on the growth response caused by unsaturated fatty acids have been noted (8,10). In an investigation of growth factors in milk for *L. bifidus* (5), it was found that milk fat contains factors required for the growth of various mutant strains of *L. bifidus* not replaceable by biotin; if a need for biotin exists the requirement is very low. Further investigation showed that the activity of milk fat was associated with the unsaturated fatty acid fraction of milk. Oleic acid (9-octadecenoic acid) and palmitoleic acid (9-hexadecenoic acid), present in human milk fat, were found to be active. It is the purpose of the present paper to report a comparison of the growth-promoting properties of oleic acid and hexadecenoic acid for two lactobacilli; *L. bifidus*, an organism for which unsaturated fatty acids are essential for growth, and *L. arabinosus*, requiring unsaturated fatty acids for growth only when biotin is absent from the medium.

## EXPERIMENTAL

### *Fatty Acids*

The sample of hexadecenoic acid used, isolated by Brown and Orians from human milk fat (6), had an iodine number of 94.8, and a neutralization equivalent of 255.5.

The oleic, stearic, and palmitic acids used were commercial samples. The fatty acids were emulsified by intimately mixing with an equal weight of gum tragacanth, diluting the mixture with water, and homogenizing. The pH of the emulsion was adjusted to 6.4, diluted to a definite concentration and autoclaved. Working solutions containing 200  $\mu\text{g./ml.}$  were prepared by diluting the stock emulsions. Control experiments showed gum tragacanth to be inactive for the growth of *L. bifidus* and *L. arabinosus*.

In experiments where the effects of unsaturated and saturated fatty acids were investigated, constant ratios of the fatty acids were used, 0.3 as much palmitic acid, and 0.15 as much stearic acid as the unsaturated acid being tested was present in each tube.

TABLE I  
*Double-Strength Basal Medium*

	<i>g</i>		<i>μg</i>
Potassium phosphate (dipotassium)	5.0	Thiamin hydrochloride	400
Sodium acetate	50.0	Riboflavin	400
Vitamin-free acid hydrolyzed casein <sup>a</sup>	10.0	Nicotinic acid	1200
		Calcium pantothenate	800
Dextrose	40.0	Pyridoxine hydrochloride	2400
L-Cystine	0.4	Folic acid	20
L-Tryptophan	0.4	p-Aminobenzoic acid	20
Adenine sulfate	0.020	Salts B	10 ml.
Guanine hydrochloride	0.020	Volume to 1000 ml. at pH 6.4	
Uracil	0.020		
Xanthine	0.020		
Asparagine	0.2		
DL-Alanine	0.4		

Composition of Salts B

	<i>g.</i>
MgSO <sub>4</sub> ·7H <sub>2</sub> O	10.0
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.5
NaCl	0.5
MnSO <sub>4</sub> ·2H <sub>2</sub> O	0.337
H <sub>2</sub> O to 250 ml.	

<sup>a</sup> General Biochemicals Incorporated, Chagrin Falls, Ohio.

CULTURES AND MEDIA

The organisms used in these experiments were *L. arabinosus* 17-5 (ATCC) and a mutant strain of *L. bifidus*.<sup>1</sup> For inoculum, *L. arabinosus* was grown in the complete

<sup>1</sup> Furnished by R. F. Norris, R. M. Tomarelli and P. Gyorgy, Hospitals of The University of Pennsylvania. The organism was originally bifid. On repeated transfer

medium (Table I), aerobically. *L. bifidus* was grown in the same medium with 10% skim breast milk added and incubated anaerobically in Brewer Anaerobe jars. Transfers were made daily, and incubation was at 35°C. Inoculations were made with the organisms centrifuged, washed, and resuspended in 0.85% saline. Two drops from a No. 22 hypodermic needle were used for the inoculation of each tube. The incubation of the inoculated test samples was at 35°C., aerobically, for 40 hr.

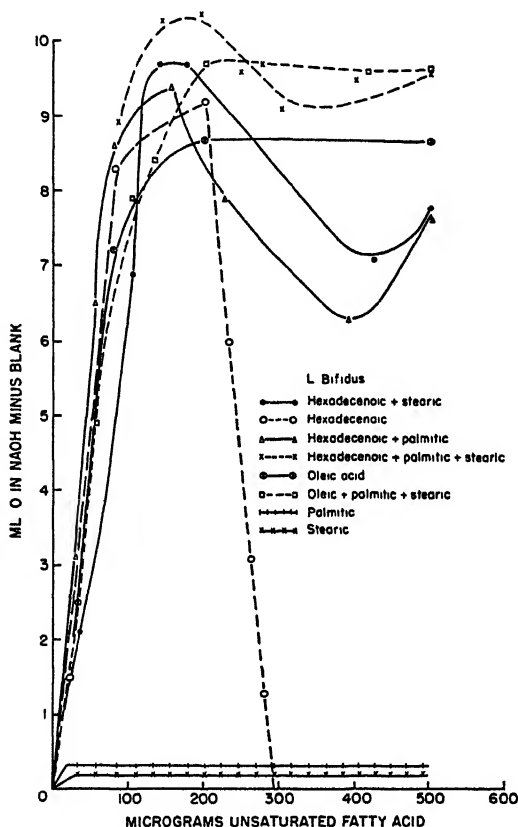


Fig. 1. The acidimetric growth response of *Lactobacillus bifidus* to fatty acids.

**Microbiological Methods.**—The usual procedures for microbiological assays were used. With both organisms 5.0 ml. of the basal medium (Table I) was diluted to a final volume of 10.0 ml. with the test sample and sterile distilled water. Growth was determined by acidimetric titration with a Beckman pH meter.

the culture had changed to rod shaped organisms indicating mutation. This mutant strain does not require inclusion of a desoxyriboside in the medium for growth as has been reported for another strain (9).

## EXPERIMENTAL RESULTS

Hexadecenoic acid and oleic acid have different effects on the growth of *L. bifidus* (Fig. 1). With this organism, hexadecenoic acid is highly active at the lower levels of addition but depresses growth entirely at slightly higher levels. This toxic effect of hexadecenoic acid is counter-

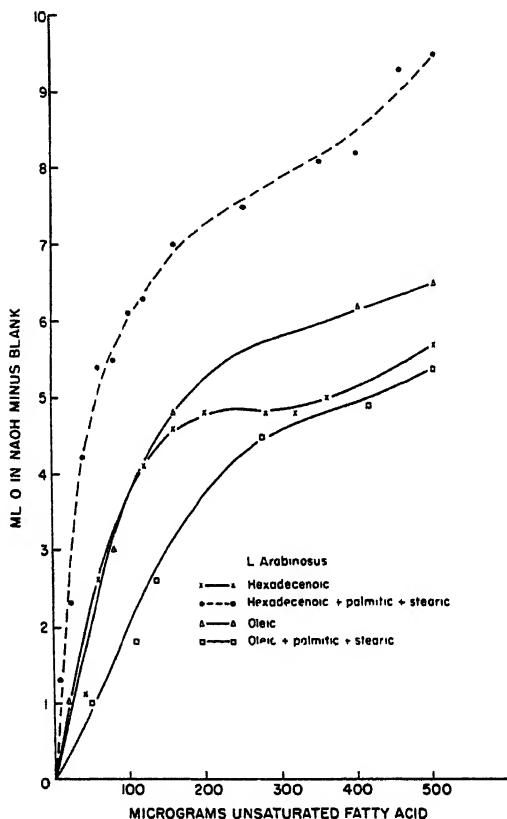


FIG. 2. The acidimetric growth response of *Lactobacillus arabinosus* to fatty acids.

acted by either palmitic acid or stearic acid and most effectively by a mixture of palmitic and stearic acids. Oleic acid does not show growth depression at higher levels of addition. We have found in other experiments that as much as 2000  $\mu\text{g.}$  of oleic acid/tube did not cause a

growth depression with *L. bifidus*. Synergism between oleic acid and a mixture of palmitic acid and stearic acid in increasing acid production is slight.

With *L. arabinosus* in a medium with no added biotin (Fig. 2), hexadecenoic acid does not cause growth depression at levels up to 500  $\mu\text{g.}/\text{tube}$ . In other experiments, levels up to 1000  $\mu\text{g.}$  of hexadecenoic acid/tube did not inhibit growth. The growth response by this organism to hexadecenoic acid and oleic acid in replacing biotin is similar. A definite synergism exists between hexadecenoic acid and a mixture of palmitic and stearic acids. In contrast to the effect of saturated fatty acids when combined with hexadecenoic acid, a slight inhibition in acid production occurred when a mixture of palmitic and stearic acids was added to the oleic acid.

### DISCUSSION

The data obtained show that hexadecenoic acid is a growth factor for certain lactobacilli with distinct differences in activity and synergistic effects of saturated fatty acids as compared to oleic acid. The mechanism of the synergism of hexadecenoic acid with the saturated fatty acids may be explained by their effect of reducing the toxicity of hexadecenoic acid. 9-Hexadecenoic acid is widely distributed in nature, being present in many naturally-occurring fats and oils (7), and probably contributes to the bacterial growth-stimulating properties of natural fatty materials (2,8). The vegetable phosphatides contain from 5-10% 9-hexadecenoic acid (7).

In considering the relationship between unsaturated fatty acid activity and structure, the data presented indicate that the length of the carbon chain is not a critical factor for activity.

### ACKNOWLEDGMENTS

We wish to express our thanks to Dr. J. B. Brown for supplying us the hexadecenoic acid used in these experiments.

### SUMMARY

Palmitoleic acid (9-hexadecenoic acid) supports good acid production of a mutant strain of *L. bifidus* at lower levels of concentration, and inhibits at higher levels, in contrast to oleic acid which supports acid production at both high and low levels of addition. With *L. arabinosus*,

hexadecenoic acid, in a medium with no added biotin, stimulates acid production in a manner similar to oleic acid.

With a mutant strain of *L. bifidus*, palmitic and stearic acids, singly, or better in combination, remove the inhibition caused by addition of higher levels of hexadecenoic acid. Palmitic and stearic acids in combination show a synergistic effect with hexadecenoic acid in growth stimulation of *L. arabinosus*.

A synergistic effect of saturated fatty acids and oleic acid on the growth of a mutant strain of *L. bifidus* has been demonstrated. With *L. arabinosus*, the growth stimulation caused by oleic acid is slightly inhibited by palmitic and stearic acids.

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# Ultracentrifugal Study of Bovine Plasma Protein Fractions

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## INTRODUCTION

Since the development of the ultracentrifuge by Svedberg, a considerable amount of study has been given to the protein constituents of serum and plasma by Mutzenbecher (1), McFarlane (2), and Pedersen (3) using whole sera of various species. In addition to his work on whole sera, Pedersen (3) has made an exhaustive study of the fractionation of sera with ammonium sulfate by following the fractionation with the ultracentrifuge. In these studies, adult human plasma and sera of the adult cow, calf, foetal calf, adult human, human umbilicus, adult rabbit, foetal rabbit, foal, and foetal sheep were fractionated by means of the classical ammonium sulfate procedures, and the fractions were examined on the ultracentrifuge. Studies were also made on the fractionation of delipidated sera of various species.

In view of the availability of a number of plasma protein fractions resulting from the application of low temperature alcoholic fractionation procedures developed in the laboratory of E. J. Cohn, an ultracentrifugal study on some of these fractions was undertaken. The protein preparations were made in the Armour Laboratories by employing essentially the methods developed by Cohn *et al.* (4,5,6). Five protein fractions from bovine plasma were submitted to ultracentrifugal study: A sample of crystalline bovine albumin prepared according to Cohn *et al.* (6), Fraction IV ( $\alpha$ -globulin and other components), Fraction III-1 ( $\beta$ -globulin), Fraction II ( $\gamma$ -globulin), and Fraction I (fibrinogen). With the exception of Fraction IV, all of the fractions consisted for the most part of one component, electrophoretically. The electrophoretic analyses were furnished by the Armour Laboratories.

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## EXPERIMENTAL

All of the ultracentrifugal studies on the bovine plasma protein fractions were made on the Svedberg oil turbine velocity centrifuge. A speed of 59,000 r. p. m. was used. The Lamm scale method for observing the sedimenting boundaries was used throughout the investigation. All the proteins were dissolved in 0.2 *M* NaCl. A few trial runs were made by using 0.12 *M* Na<sub>2</sub>HPO<sub>4</sub>. The protein preparations were usually in the lyophilized form and stored under refrigeration. Sedimentation runs were made at ten different protein concentrations ranging from 0.15% to 5.00% based on the dry weight of the preparations. By determining the sedimentation constants at a number of different concentrations, the variation of sedimentation constant with protein concentration could be observed. The treatment of the ultracentrifugal data was essentially that of Svedberg and Pedersen (7) and Pedersen (3).

## RESULTS

*Fraction I (Fibrinogen), Lot No. C185*

This preparation contains about 40% sodium citrate. Figure 1 shows the sedimentation diagram for fibrinogen in a 0.5% solution after

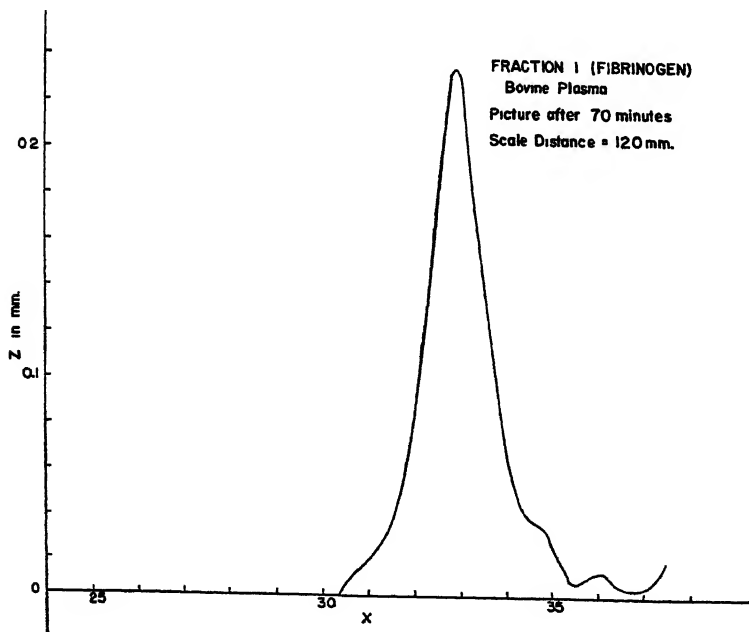


Fig. 1. Sedimentation diagram for Fraction I (fibrinogen).

running for 70 min. The ordinate,  $Z$ , represents the scale line displacement and is proportional to the concentration gradient, while the abscissa,  $x$ , is the scale reading for the test run and is proportional to the distance from the axis of rotation, meniscus, etc. The diagram gives evidence of having a small amount of a heavier component. The sharp-

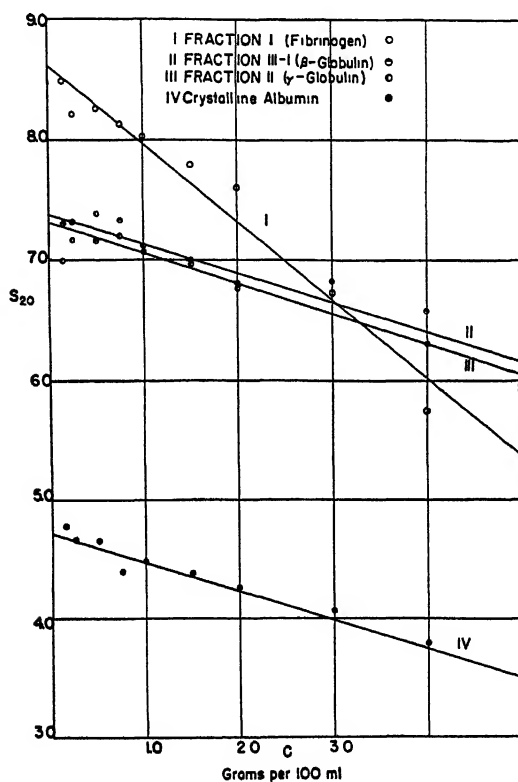


FIG. 2. Regression lines for sedimentation constant on concentration.

ness of the curve is the result of the low diffusion constant of fibrinogen. The diagram represents a rather good preparation of fibrinogen. Curve I in Fig. 2 illustrates the variation of the sedimentation constant with change in concentration of protein. The concentration here refers to grams of Fraction I/100 ml. of 0.2  $M$  NaCl. The equation of the line of regression for  $S_{20}$  on concentration as calculated by the method of least

TABLE I

Protein fraction	Line of regression for $S_{20}$ on concentration	Correlation coefficient $r$	Line of regression for $S_{20}$ on $\Delta n$	Correlation coefficient $r$
I	$S_{20} = 8.62 - 0.65c$	-0.99	$S_{20} = 8.43 - 0.0052\Delta n$	-0.96
II	$S_{20} = 7.31 - 0.25c$	-0.95	$S_{20} = 7.28 - 0.0016\Delta n$	-0.90
III-1	$S_{20} = 7.37 - 0.24c$	-0.95	$S_{20} = 7.37 - 0.0020\Delta n$	-0.97
IV				
Heavy comp.	$S_{20} = 19.45 - 1.22c$	-0.97	$S_{20} = 19.68 - 0.041\Delta n$	-0.95
Second comp.	$S_{20} = 7.46 - 0.38c$	-0.87	$S_{20} = 7.31 - 0.0072\Delta n$	-0.51
Third comp.	$S_{20} = 5.76 - 0.37c$	-0.92	$S_{20} = 5.64 - 0.0047\Delta n$	-0.87
Cryst. albumin	$S_{20} = 4.73 - 0.25c$	-0.99	$S_{20} = 4.73 - 0.0016\Delta n$	-0.99

squares and the correlation coefficient are given in Table I. Concentrations in terms of  $\Delta n$ , the refractive index increment, are calculated from the diagram by the method of Pedersen (7). The equation for the line of regression for  $S_{20}$  on  $\Delta n$ , obtained by the method of least squares, and the correlation coefficient are given in Table I.

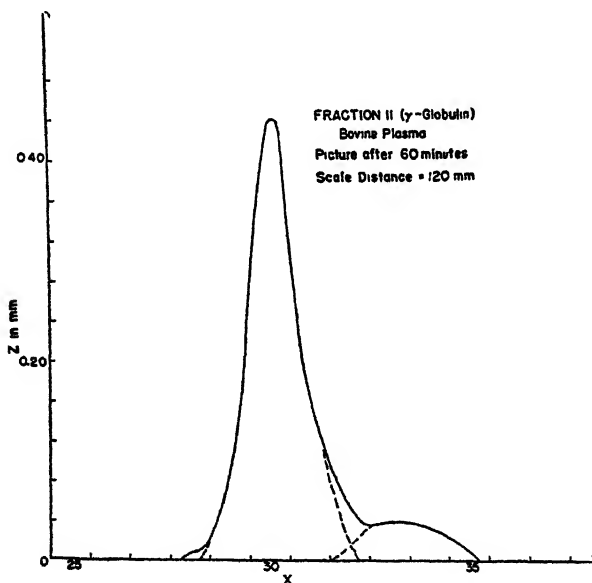


FIG. 3. Sedimentation diagram for Fraction II ( $\gamma$ -globulin).

It was not possible to determine  $\Delta n$  for the higher concentrations because the concentration gradients were so great that *schlieren* values were obtained instead of scale line displacements suitable for constructing Gaussian distribution curves.

*Fraction II ( $\gamma$ -Globulin), Lot No. 174IV*

The sedimentation diagram for a 0.5% solution after sedimenting for 60 min. is shown in Fig. 3. The diagram gives evidence of about 18% of

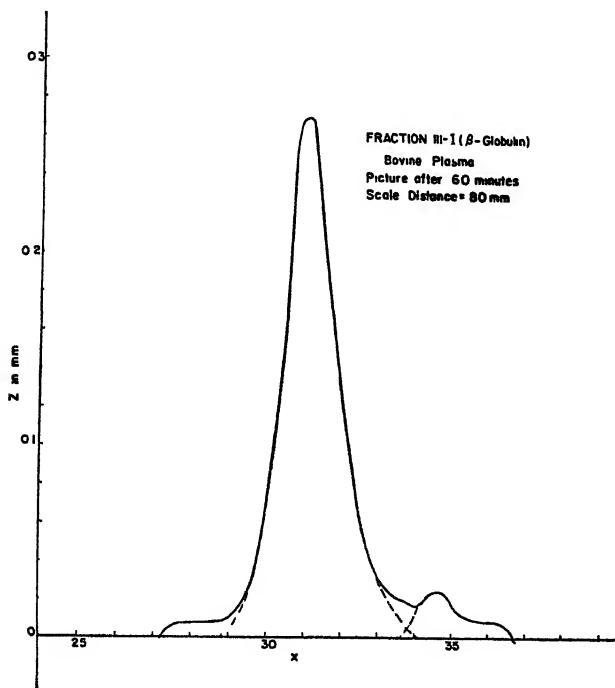


FIG. 4. Sedimentation diagram for Fraction III-1 ( $\beta$ -globulin).

a heavier component sedimenting at 10.85 *S*. There seems to be very little if any lighter material. Curve III of Fig. 2 shows the variation of the sedimentation constant with change in concentration. The equations for the lines of regression of  $S_{20}$  on concentration and on  $\Delta n$  together with the respective correlation coefficients are given in Table I.

*Fraction III-1 ( $\beta$ -Globulin), Lot No. 174W*

The sedimentation diagram for a 0.5% solution after sedimenting for 60 min. is shown in Fig. 4. The presence of at least two heavier components amounting to about 13% and a very small quantity of a lighter component is evident from the diagram. The heavier material seems to have an approximate sedimentation constant of 10.43 S. The regression line for the dependence of  $S_{20}$  on concentration is given by Curve II

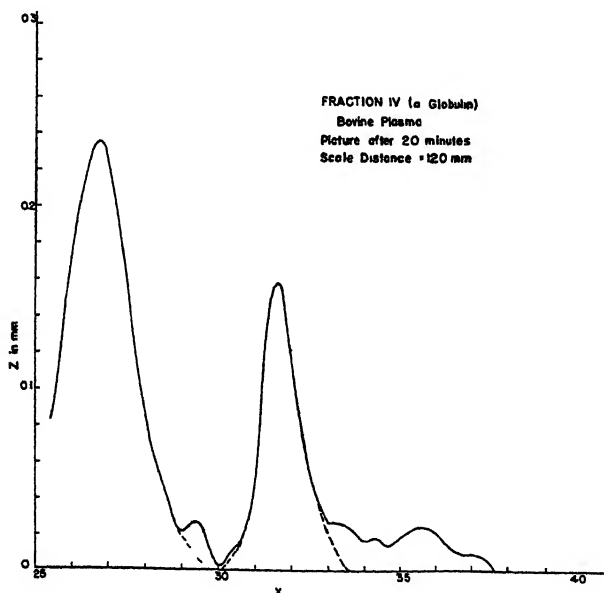


FIG. 5. Sedimentation diagram for Fraction IV.

in Fig. 2. The equations for the lines of regression relating  $S_{20}$  to concentration and to  $\Delta n$  together with the respective correlation coefficients are given in Table I.

*Fraction IV ( $\alpha$ -Globulin), Lot No. C189*

Figure 5 gives the sedimentation diagram for a 0.5% solution after sedimenting for 20 min. Obviously this is a complex mixture of proteins and consists of at least two main components with an indication of smaller amounts of other components. As the sedimentation continues,

the slower main component breaks up into at least two separate components as shown in Fig. 6, a diagram after 60 min. of sedimentation. Each of these three main components was treated separately as to the dependence of its respective sedimentation constant upon concentration.

Fig. 7 shows the regression line for the dependence of the  $S_{20}$  of the heavy component upon its concentration in solution. The concentration on the abscissa represents the total concentration of protein and not the concentration of the individual component—that can only be obtained from the diagram. It was assumed that the total concentra-

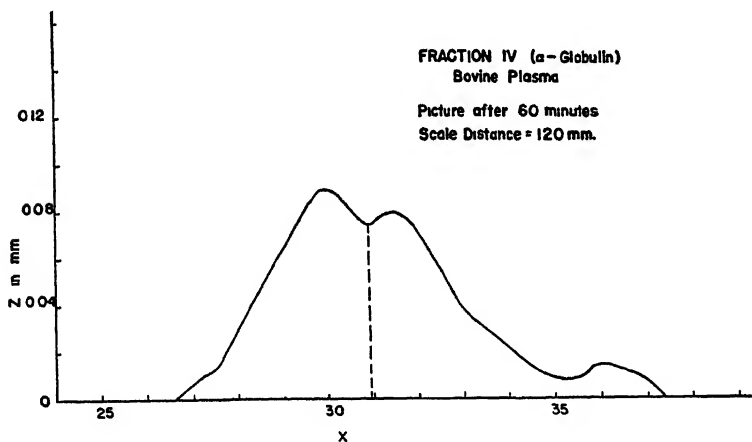


FIG. 6. Sedimentation diagram for Fraction IV showing split of slower main component.

tion should be multiplied by some arbitrary constant in order to arrive at the concentration of the individual component. The  $S_{20}$  value at zero concentration as determined by means of the method of least squares would not be altered by multiplication with such a constant. Table I gives the equations for the lines of regression relating  $S_{20}$  to concentration and to  $\Delta n$  together with the respective correlation coefficients.

The regression line for the dependence of the  $S_{20}$  of the second component upon its concentration in solution is given in Fig. 7. Here again, as for the heavy component, the concentration on the abscissa repre-

sents the total concentration of protein. The same assumption was made regarding this matter as was made for the heavy component. Equations for the lines of regression defining the dependence of  $S_{20}$  on concentration and on  $\Delta n$  with the respective correlation coefficients are given in Table I. The low values of the correlation coefficients are in part due

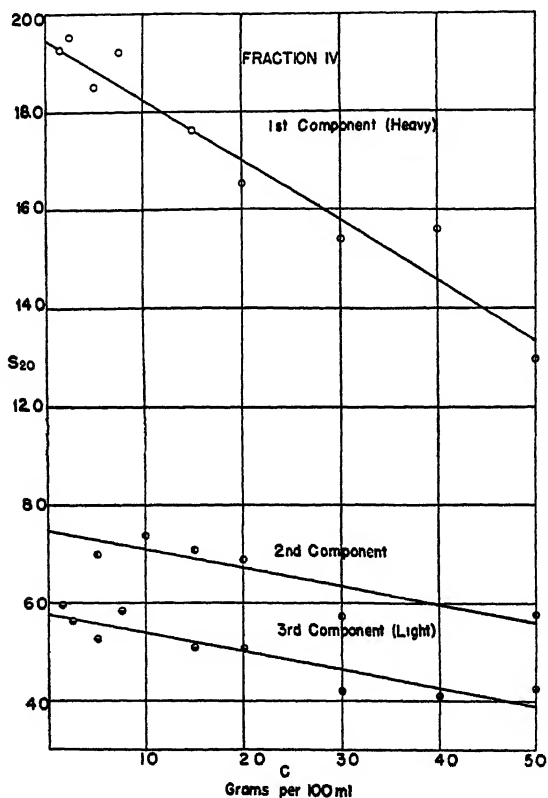


FIG. 7. Regression lines for sedimentation constant on concentration for components of Fraction IV.

to the small amount of data available inasmuch as splitting of the components did not take place at all concentrations.

Figure 7 shows the regression line for the dependence of  $S_{20}$  of the third component (light component) upon its concentration in solution. The concentration represents total protein concentration. The same

assumption was made here as was made for the concentrations of the previous components. The equations for the lines of regression relating  $S_{20}$  to concentration and  $\Delta n$  together with the respective correlation coefficients are given in Table I.

*Crystalline Albumin, Lot No. 46*

The sedimentation diagram for a 0.5% albumin solution after running 100 min. is given in Fig. 8. While this protein is probably one of

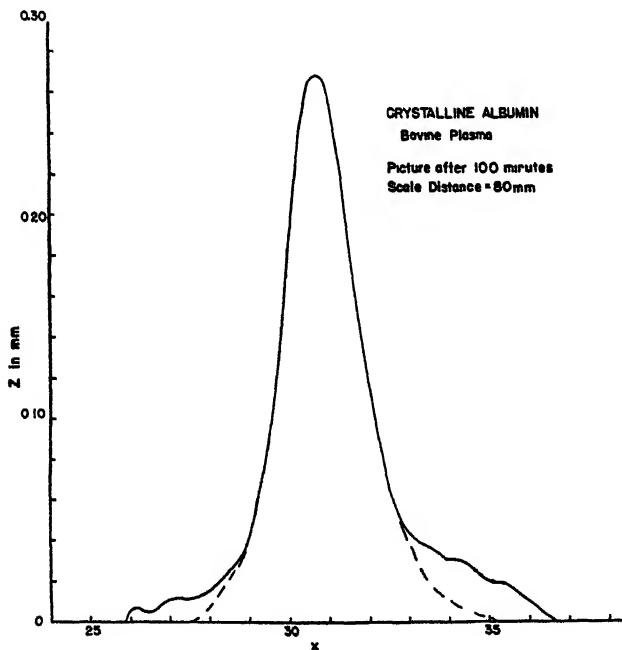


FIG. 8. Sedimentation diagram for crystalline albumin.

the purest of the plasma proteins isolated, there is evidence of small quantities of nondescript heavier components and possibly a very small amount of lighter material present. Curve IV of Fig. 2 illustrates the influence of protein concentration on  $S_{20}$ . In Table I, are found the equations relating  $S_{20}$  to concentration and to  $\Delta n$  together with the respective correlation coefficients.

Table II summarizes the  $\Delta n$  data.

TABLE II  
Summary of  $\Delta n$  Data

Concentration in %	Fraction I		Fraction II		Fraction III-1		Crystalline albumin		Fraction IV							
									Heavy component		Second component		Third component			
	$S_{20}$	$\Delta n$	$S_{20}$	$\Delta n$	$S_{20}$	$\Delta n$	$S_{20}$	$\Delta n$	$S_{20}$	$\Delta n$	$S_{20}$	$\Delta n$	$S_{20}$	$\Delta n$	$S_{20}$	$\Delta n$
0.15	8.48	12	6.99	26	7.30	22	4.78	24	19.26	8	—	—	5.96	13		
0.25	8.21	20	7.16	43	7.32	33	4.66	40	19.52	10	—	—	5.63	23		
0.50	8.26	40	7.38	68	7.16	67	4.65	76	18.51	18	6.99	16	5.26	21		
0.75	8.13	64	7.20	101	7.33	94	4.39	104	19.21	31	—	—	5.83	64		
1.00	8.03	76	7.12	128	7.07	125	4.48	159	17.73	30	7.38	22	5.42	40		
1.50	7.79	108	6.97	183	6.99	176	4.38	212	17.63	48	7.09	35	5.09	70		
2.00	7.61	168	6.77	262	6.81	223	4.26	295	16.54	84	6.89	50	5.08	97		
3.00	6.73	—	6.73	417	6.83	329	4.06	454	15.41	116	5.73	—	4.21	270		
4.00	5.75	—	6.31	544	6.53	473	3.79	613	15.60	123	—	—	4.12	203		
5.00	5.40	—	5.92	—	5.96	659	3.45	785	12.98	131	5.77	—	4.25	380		

## DISCUSSION

The values for the sedimentation constant of Fraction I extrapolated to zero concentration and zero  $\Delta n$  agree rather well: namely, 8.62 and 8.43, respectively. Although the concentration values represent grams of Fraction I/100 ml. rather than true protein concentrations because of the presence of 40% sodium citrate, the  $\Delta n$  values should give a pretty good indication of true protein concentration. The values of the intercepts are close considering the accuracy of the methods involved. The slopes of the two lines are different as would be expected, the one equal to the other multiplied by some constant relating concentration to index of refraction. The correlation coefficients, 0.99 and 0.96, indicate that the straight-line relation holds. The relatively high slope indicates a rather large dependence of  $S_{20}$  on concentration. This would be expected in view of the long fibrous nature of the fibrinogen molecule.

The good agreement between the  $S_{20}$  values at zero concentration and zero  $\Delta n$  for Fraction II with the expected difference in slopes shows again that absolute units of concentration are not important as long as  $S_{20}$  is carefully determined for known arbitrary units of concentration. The correlation coefficients, 0.95 and 0.90, indicate the straight-line

relationship. With change in concentration the change in  $S_{20}$  is not as great as for Fraction I.

The discussion concerning Fraction II is applicable to Fraction III-1. The  $S_{20}$  value at zero concentration and zero  $\Delta n$  are the same and agree with  $S_{20}$  for Fraction II. The molecular sizes and shapes must be about the same. The slopes of the equations indicate rather small concentration dependence—at least not nearly as great as that found for Fraction I. The  $r$  values, 0.95 and 0.97, indicate a good adherence to the straight-line relationship.

Fraction IV presents a protein mixture rather than any preponderance of a single component. The heavy component presents rather high concentration dependence (as judged by the slope of the regression line)—even higher than for fibrinogen. This heavy component is probably the 20-component described by Pedersen (3). The concentration dependence can be due to a variety of things inasmuch as a mixture is dealt with here. For one thing, this dependence might indicate a long filamentous molecule as well as a viscosity effect due to the presence of the other components. The  $S_{20}$  values at zero concentration and zero  $\Delta n$  agree well, and the  $r$  values, 0.97 and 0.95, indicate the straight-line relationship.

The second component presents less concentration dependence, and due to the absence of resolution at all concentrations, there are fewer data available for an accurate determination of  $S_{20}$  at zero concentration and zero  $\Delta n$ . The agreement is good considering these factors. The  $r$  values are not high because of insufficient data, however, they do indicate a trend for the straight-line relationship.

The third component presents rather good agreement between the  $S_{20}$  values for zero concentration and zero  $\Delta n$ , respectively. The concentration dependence is about the same as for the second component. The  $r$  values are better than those for the second component because of more and better data.

The excellent agreement between the  $S_{20}$  value at zero concentration and that at zero  $\Delta n$  for crystalline albumin is to be expected in view of the near approach to a pure protein. The  $r$  values are likewise indicative of the straight-line relationship between sedimentation and concentration.

When the sedimentation constant-concentration relationship was fitted to a curve of the second degree, there was no statistical advantage over the straight-line fit. The plots were used chiefly to obtain the

sedimentation value at zero concentration. There was no significant difference between the intercepts of the second degree curves and the straight-lines.

#### ACKNOWLEDGMENTS

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#### SUMMARY

An ultracentrifugal study on a series of protein fractions from bovine plasma has been made. By extrapolating to zero concentration the straight line formed by plotting sedimentation against concentration, the sedimentation constants at infinite dilution have been determined. The following values were obtained: fibrinogen, 8.43–8.62 *S*;  $\gamma$ -globulin, 7.28–7.31 *S*;  $\beta$ -globulin, 7.37 *S*; a mixture of  $\alpha$ -globulins, 19.45–19.68 *S*, 7.31–7.46 *S*, and 5.64–5.76 *S*; albumin, 4.73 *S*. The homogeneity of the various preparations was discussed.

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# Transosazonation

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## INTRODUCTION

A relatively large amount of research has been concerned with the possibility of detaching the hydrazine groups from osazones. This is the basis of osone formation. The correct formulation of the reaction is complicated by the existence of at least 20 geometrical isomers and the likelihood of both cyclic and acyclic structures (1, 2). If the osazones are written in the conventional form as open chain bishydrazones of the dicarbonyl compounds, cleavage by means of hydrochloric acid (3, 4) represents a hydrolysis of the azomethine groupings, analogs of Schiff's bases, while cleavage by benzaldehyde (5, 6, 7) or its homologs is due to competition between carbonyl residues.

Very little is known about reactions of osazones in which hydrazine residues are exchanged, for which the term "transosazonation reactions" is proposed. The first example of a transosazonation reaction in the sugar group was given by Neuberg (8) who described the preparation of L-arabinose *p*-bromophenylosazone by warming a solution of L-arabinose phenylosazone with *p*-bromophenylhydrazine acetate. Further investigations of the phenomenon were made by Votoček and Vondráček (9). Having studied the action of various hydrazines on hydrazones they found that in osazones either one or both groups may be exchanged. Heating 1 mole glyoxal bisphenylhydrazone with 10 moles methylphenylhydrazine acetate in alcoholic solution they obtained exclusively glyoxal bismethylphenylhydrazone; but heating an alcoholic solution of 1 mole glucose phenylosazone with 10 moles methylphenylhydrazine and 10 moles acetic acid produced the mixed osazone  $\text{CH}[:\text{N}.\text{N}(\text{CH}_3)\text{C}_6\text{H}_5]—\text{C}(:\text{N}.\text{NHC}_6\text{H}_5)—(\text{CHOH})_4—$

<sup>1</sup> From a thesis submitted to the Graduate Faculty of the Polytechnic Institute of Brooklyn, June 1947. Presented at the A. C. S. meeting, New York, Fall 1947.

$\text{CH}_2\text{OH}$ . Oddo and Cesaris (10) under the same conditions obtained the isomeric osazone  $\text{CH}(:\text{N}.\text{NHC}_6\text{H}_5) - \text{C}[:\text{N}.\text{N}(\text{CH}_3)\text{C}_6\text{H}_5] - (\text{CHOH})_3 - \text{CH}_2\text{OH}$ . Mixed osazones were obtained when Votoček and Valentin (11, 12) treated fructose methylphenylosazone with an excess of *p*-bromophenylhydrazine; but reaction of the fructose methylphenylosazone with phenylhydrazine acetate produced ordinary glucose phenylosazone. Only mixed osazones were obtained by Engel (13) working under atypical experimental conditions.

## RESULTS

While a weak linkage between hydrazine and sugar residue may be postulated (9), nothing definite is known about the mechanism of such transosazonation reactions. It was hoped that by selecting substituted hydrazines specially well adapted as transosazonizing agents some insight into this problem might be gained.

The insolubility of the 2,4-dinitrophenylosazones makes them extremely well suited for the purpose. Better yields may be expected and there is greater likelihood of complete reaction, leading to pure products instead of the mixed osazones obtained by most previous workers. In addition to these advantages, a sensitive colorimetric test (see below) is available for the detection and determination of nitrophenylosazones.

Prior to this, nitro substituted phenylhydrazines had never been used for transosazonations. They were used exclusively in the present study. Transosazonations with 2,4-dinitrophenylhydrazine are made in 2 *N* HCl solution. This procedure can be applied whenever the sugars involved or their osazones resist HCl. When this is not the case, as, e.g., with erythrosazone, pentosazones, and disaccharide osazones, *p*-nitrophenylhydrazine in acetic acid solution can be used. Disaccharide osazones are hydrolyzed to monosaccharides and monosaccharide osazones by HCl; tetra- and pentosazones have a tendency to decompose. All these substances, however, are stable towards acetic acid.

2,4-Dinitro- and *p*-nitrophenylosazones can be detected in small amounts by a sensitive colorimetric test. Unlike ordinary phenylosazones, methylphenylosazones, bromophenylosazones, etc., the corresponding *p*-nitro compounds give a deep indigo blue coloration with alcoholic KOH or NaOH, while 2,4-dinitrophenylosazones turn to a violet shade, resembling methyl violet. The qualitative color test described by Gnehm and Benda (14) in 1896 and amplified by Bamberger

(15) and Hyde (16) has recently been developed for quantitative determinations by Neuberg and Strauss (17). The color is probably due to formation of the aci-nitro compounds. It is sufficiently sensitive to determine transosazonations of microgram quantities, *i.e.*, 1  $\mu$ g. of transosazonized phenylosazone, by absorption on kaolin and elution with  $\text{NaOC}_2\text{H}_5$ .

The test further allows a demonstration of the extraordinarily rapid start of the transosazonation reaction: If a clear solution of excess 2,4-dinitrophenylhydrazine in 2 *N* HCl is added to 0.1 g. glucosazone, dissolved in a few drops of pyridine or alcohol or even to the fine powder, a clear solution momentarily appears on warming, but in less than 1 min. precipitation of the red glucose 2,4-dinitrophenylosazone begins. By means of the color produced with  $\text{NaOC}_2\text{H}_5$ , the presence of *p*- or dinitrophenylosazones of methylglyoxal, diacetyl, glyceraldehyde, erythrose, arabinose, glucose, lactose, and maltose could be shown after heating for 1 min. or less. For these qualitative experiments, methylglyoxal bis-*p*-bromophenylhydrazine, diacetyl 2,5-bis(dichlorophenyl)hydrazine and the ordinary phenylosazones of the mentioned sugars were used.

Reaction times and experimental conditions had to be modified according to the nature of the osazones used. While the start of the reaction could be easily determined colorimetrically, it was less simple to assure the absolute purity of the nitrated osazones. Osazones labeled with halogen atoms were used as starting materials so that the course of the reaction could be followed and the presence of any unchanged starting material or intermediately-formed mixed osazones easily detected. Most of the 2,5-dichloro- and *p*-bromophenylosazones prepared for this purpose have not previously been described, *i.e.*, diacetyl bis-2,5-dichlorophenylhydrazine, L-sorbose 2,5-dichlorophenylosazone, cellobiose 2,5-dichlorophenylosazone, glyoxal bis-*p*-bromophenylhydrazine, methylglyoxal bis-*p*-bromophenylhydrazine, DL-erythrose *p*-bromophenylosazone, D-galactose *p*-bromophenylosazone, and D-glycero-D-guloaldoheptose *p*-bromophenylosazone. Others had only been made from osones previously, *i.e.*, maltosone and lactosone bis-*p*-bromophenylhydrazones (5, 6). Glycerose *p*-bromophenylosazone was prepared from oxidized glycerol instead of pure glyceraldehyde as by Wohl and Neuberg (18).

When transosazonation was believed to be complete, the product was tested for the presence of organically bound halogen. For the many

halogen determinations that had to be made it was possible to employ a procedure much simpler than those usually used. This almost forgotten method by Mandel and Neuberg (19) is based on the action of strong  $\text{H}_2\text{O}_2$  in the presence of a trace of ferric nitrate. If  $\text{AgNO}_3$  is added to a solution of the osazone in glacial acetic acid, nitric acid, and  $\text{Fe}(\text{NO}_3)_3$ , and the halogen then liberated, even smallest traces can be determined.

Since halogen-free nitrophenylosazones were obtained from all the substituted osazones subjected to transosazonation, the fairly general applicability of the reaction and the formation of pure products can be claimed. Results indicate that the reaction applies not only to the osazones of all sugars in the usual sense of the term, but also to substances which can be regarded as substitution products of glyoxal. The latter include the bishydrazones of methylglyoxal,  $\text{CH}_3-\text{C}(:\text{N}.\text{NHC}_6\text{H}_5)-\text{CH}(:\text{N}.\text{NHC}_6\text{H}_5)$  and of diacetyl,  $\text{CH}_3-\text{C}(:\text{N}.\text{NHC}_6\text{H}_5)-\text{C}(:\text{N}.\text{NHC}_6\text{H}_5)-\text{CH}_3$ , although diacetyl clearly does not have a terminal CO group.<sup>2</sup> Only the osazone of dioxosuccinic acid could not be transosazonized. This appears reasonable in view of the pyrazolone ring structure of tartrazine which prevents interaction with the transosazonizing agent.

It was also possible to devise experiments giving a fair idea of the mechanism. The transosazonizing agent was omitted in an otherwise identical acetic acid solution of the osazones. After heating, the solution was centrifuged in the presence of bentonite or kaolin to absorb the unchanged starting material, and the supernatant liquid was then decolorized with charcoal. An absolutely clear and colorless liquid resulted which, however, gave positive tests for osone. This is the more surprising since the great stability of the osazones of the lower sugars, even toward concentrated hydrochloric acid, has been known for a long time. Emil Fischer (4) stated that under the influence of concentrated HCl, erythrosazone and glycerosazone were first converted into hydrochlorides, then, on heating, decomposed, but no phenylhydrazine was split off. Glyoxal bis-phenylhydrazine and concentrated HCl even yielded a stable crystalline reddish yellow hydrochloride (21); tartrazine was not attacked at all (4), even by boiling HCl. Will (22) added

<sup>2</sup> Like glyoxal, methylglyoxal and diacetyl have frequently been found as cleavage products of higher sugars. A direct connection between diacetyl and a carbohydrate, *i.e.*, streptobiosamine, has recently been demonstrated by Brink, Kuehl, Flynn, and Folkers (20).

hydroxypyruvic acid osazone to the list of osazones forming a stable hydrochloride with HCl. According to Nastvogel (23), this osazone dissolves in strong HCl with as much difficulty as in water. More recently den Otter (24) confirmed the stability of glycerosazone toward HCl and  $\text{H}_2\text{SO}_4$ , nor could he hydrolyze this osazone with benzaldehyde or glucose, even in the presence of HCl or acetic acid.

In contrast to this remarkable stability, it was now possible to establish in an unambiguous manner that glucose phenylosazone and xylose phenylosazone are hydrolyzed to the corresponding osones to the extent of 4-7% in acetic acid solutions. This is particularly interesting since the osazone reaction is ordinarily carried out in acetic acid solution. On the other hand, this reversible formation of osones from the osazone under the conditions of normal osazone formation appears to be at least partly responsible for the low yields normally obtained. Thus Wolfrom (25) obtained 34% fructose phenylosazone, although Riebsomer (26), in contrast to other workers, surprisingly claimed quantitative yields. Only recently, Neuberg and Strauss (27) succeeded in making the osazone reaction a quantitative one. This was made possible by the use of 2,4-dinitrophenylhydrazine and more prolonged heating. The reason why the reaction can proceed to completion in the case of 2,4-dinitrophenylhydrazine lies in the comparative insolubility of the osazones formed.

Briefly, the transosazonation reactions studied appeared to take the following course: The osazone in acid solution was hydrolyzed to a small extent to form the equilibrium concentration of osone. The osone produced, at once reacted with the nitrophenylhydrazine present and the practically insoluble nitrophenylosazone precipitated out. Osone continued to form in small amounts until the entire substrate was converted to the much less soluble nitrophenylosazone by consecutive equilibrium shifts.

While proven in the case of D-xylosazone and D-glucosazone, this scheme must also apply to the osazones of lower sugars and the related methylglyoxal and diacetyl compounds. In spite of the great stability toward hydrolyzing agents mentioned above, their transosazonation is possible under the given experimental conditions, so that in this case, too, hydrolysis to the corresponding dicarbonyl derivative appears to take place to an exceedingly small extent, the nitrophenylosazones again precipitating due to their comparative insolubility. An analog to this process can be found in the literature: The bissemicarbazone of

methylglyoxal is insoluble in water and most solvents and resembles osazones in many respects. Nevertheless Neuberg and Kobel (28) succeeded in converting it to the corresponding *p*-nitrophenylosazone by an exchange reaction in hot HCl solution.

In conclusion it can be said that the osazones do not exhibit the great stability toward acids generally ascribed to them. Even in acetic acid solution some osone is produced and its formation furnishes an explanation of the described phenomenon of transosazonation.

## EXPERIMENTAL

### *A. Preparation of the Osazones*

#### 1. Preparation of *p*-Bromo and 2,5-Dichlorophenylosazones

*Glyoxal bis-p-bromophenylhydrazone* (glycolaldehyde *p*-bromophenylosazone). Three g. pure *p*-bromophenylhydrazine HCl were dissolved in about 25 ml. water, 2 g. sodium acetate in 5 ml. water was added, and a few drops acetic acid and alcohol to decrease the surface tension. The clear solution was mixed with 0.6 ml. 25% glyoxal. The large excess of *p*-bromophenylhydrazine is necessary to counteract the possibility of hydrazone formation. The osazone precipitated at once at room temperature. After filtering by suction, the mother liquor must be tested to assure the presence of excess hydrazine. This is most conveniently done by adding a drop of benzaldehyde solution. The osazone was recrystallized from aqueous dioxane (needles). Smaller needles are obtained from aqueous methyl cellosolve. Yields are almost quantitative. It decomposes at 232°C.

*Anal.* Calcd.: N, 14.1. Found: N, 14.0.

*Methylglyoxal bis-p-bromophenylhydrazone* (lactic acid aldehyde or acetol *p*-bromophenylosazone). 9 ml. propylene glycol and 14.5 g. anhydrous  $\text{Na}_2\text{CO}_3$  were dissolved in 100 ml. water. This was oxidized by adding 5.5 ml. bromine in an ice-bath, keeping 45 min. in the ice-bath, then 10 min. at room temperature, just neutralizing with HCl, decolorizing with sodium metabisulfite and adding some sodium acetate to lower the acidity. The solution was made up to 200 ml. Of this, 120 ml. were utilized for the next step. Thirty-three g. *p*-bromophenylhydrazine, HCl dissolved in water, 20 g. sodium acetate, also in solution, and a few drops acetic acid were added to the methylglyoxal solution. After heating on the water-bath for 1 hr. the reaction was completed in the incubator at 37°. The compound finally obtained is extremely soluble in toluol, methyl cellosolve and dioxane. It can be recrystallized from aqueous methyl cellosolve or, better, aqueous dioxane, yielding needle clusters. It decomposes at 183°.

*Anal.* Calcd.: N, 13.6. Found: N, 13.3.

Toward the end of this study, pyruvic aldehyde had become commercially available from Carbide and Carbon Chemical Corp. The same product can be obtained by simple interaction with the hydrazine analogous to the preparation of the glycolaldehyde osazone.

*Glyceraldehyde p-bromophenylosazone*. 7.6 ml., equivalent to 9.2 g. glycerol, was dissolved with 14.5 g. anhydrous  $\text{Na}_2\text{CO}_3$  in 100 ml. water and oxidized with 5.5 ml.

bromine, exactly as in the case of propylene glycol. The solution obtained was again made up to 200 ml., and 125 ml. utilized for the formation of the bromophenylosazone. Thirty-three g. *p*-bromophenyl hydrazine, 20 g. sodium acetate, and some acetic acid, all clearly dissolved, were added to the glycerose mixture. Since some methylglyoxal could be present, the first turbidity was filtered off, and the remainder allowed to react for 48 hr. in the incubator at 37°. This mode of preparation produced the same osazone as was obtained from pure glyceraldehyde by Wohl and Neuberg (18).

*DL-Erythrose p-bromophenylosazone.* Six g. erythritol and 7.25 g. anhydrous  $\text{Na}_2\text{CO}_3$ , dissolved in 50 ml. water were cooled to + 6°, and 2.75 ml. bromine added under constant shaking in an ice-bath. After standing 45 min. at + 6°, 15 min. at 12°, and being carefully acidified with 20% HCl and decolorized with bisulfite, the solution was made up to 110 ml. To half of this solution, i.e., 55 ml., a clear solution of 16.5 g. *p*-bromophenylhydrazine HCl and 10 g. sodium acetate was added. The first, somewhat oily, precipitate was filtered off, some more bisulfite added, and the reaction completed in the incubator at 37°. Forty-eight hr. were allowed to elapse before filtering off by suction, washing, etc. Even then, yields were low. The product may be recrystallized from pyridine and acetic acid, dioxane and water, methyl cellosolve and water, aqueous methanol, benzol, or benzol and petroleum ether, giving needles in all cases. Aqueous acetone was preferred and produced long beautiful prisms. The osazone decomposes at 182°.

*Anal.* Calcd.: N, 12.3. Found: N, 12.0.

*D-Xylose, L-arabinose and D-glucose p-bromophenylosazones.* These were prepared according to the directions of Neuberg (8).

*D-Galactose p-bromophenylosazone.* A solution of 1.8 g. galactose and 6.9 g. *p*-bromophenylhydrazine HCl in 70 ml. water, plus 4.1 g. sodium acetate and 5 ml. glacial acetic acid were heated on the water-bath. After 8 hr. yellow crystals had formed and were filtered off; but the main quantity crystallized from the mother liquor after further heating on the water-bath for 3 days, and adding water and 10% acetic acid as the heating was prolonged. A combined yield of ca. 62% could be obtained from these two portions, but no further pure osazone from the second mother liquor. The osazone is soluble in methanol, ethanol, ethyl acetate, acetone, ethyl ether, dioxane, and tetrahydrofuryl alcohol; it is insoluble in benzol, petroleum ether, and chloroform. It can be recrystallized from dilute alcohol by first dissolving in methanol or ethanol, then decolorizing, and then adding hot water; if the alcoholic solution is filtered directly into hot water, resinous substances precipitate. It decomposes at 182–184°.  $[\alpha]_D$  in pyridine-absolute alcohol (4:6) = + 30°.<sup>3</sup>

*Anal.* Calcd.: N, 10.8. Found: N, 11.2.

*D-Glycero-D-guloaldoheptose p-bromophenylosazone.* 1.05 g. glucoheptose was added to a clear solution of 3.5 g. *p*-bromophenylhydrazine HCl, and 2.1 g. sodium acetate dissolved in 100 ml. water, 5 ml. 10% acetic acid, and 10 ml. alcohol. After warming on the water-bath, formation of the osazone could be ascertained after half an hour on cooling a small sample. Heating was continued for 2 hr. and the product left in the

<sup>3</sup> All polarisations were carried out in the conventional pyridine-alcohol mixture according to Neuberg (8).

<sup>4</sup> I am highly indebted to Dr. H. S. Isbell of the National Bureau of Standards for a sample of the D-glycero-D-guloaldoheptose.

refrigerator overnight. The orange-colored raw osazone was recrystallized from aqueous methanol. It can also be obtained from aqueous acetone or precipitated by petroleum ether from ethyl acetate. Cellosolve and water give a pale yellow but amorphous product. Yield: 52% and a less pure product from the mother liquor. Decomposes 203–205°.  $[\alpha]_D$  in pyridine–absolute alcohol (4:6) = + 15°.

*Anal.* Calcd.: N, 10.2. Found: N, 10.3.

*Maltose p-bromophenylosazone.* 7.2 g. maltose was added to a solution of 15 g. *p*-bromophenylhydrazine HCl and 8.8 g. sodium acetate in water, containing some 25% acetic acid. The oily substances precipitating out at first were filtered off, bisulfite was added, and the reaction continued, first in the incubator for 32 hr., then, since no osazone appeared to be formed, on the water-bath. Prolonged heating yielded the desired product, which was filtered off in the usual way and recrystallized from acetone and water, dilute alcohol or ethyl acetate. Though this osazone has been described, it had been prepared only from the osone by Fischer (5,6); Hofmann (29) stated that he had been unable to get anything but syrupy hydrazones from maltose in both neutral and acetic acid solutions.

*Lactose p-bromophenylosazone.* As in the case of maltose, this osazone had been obtained only via the osone until now; Hofmann (29) recovered the unchanged sugar when he reacted lactose with *p*-bromophenylhydrazine. The desired product has now been obtained after 24 hr. in the incubator at 37°. 6.7 g. *p*-bromophenylhydrazine HCl was dissolved in the usual way; 4.1 g. sodium acetate, a few drops 10% acetic acid and after filtering, 3.6 g. lactose in aqueous solution was added. The reaction was allowed to proceed in the incubator for 24 hr. before filtering. Further heating of the mother liquor on the water-bath increased the yield to about 60% (raw), *in toto*.

*Diacetyl-bis-2,5-dichlorophenylhydrazone* (acetyl-methylcarbinol-2,5-dichlorophenylosazone). Eight g. 2,5-dichlorophenylhydrazine was dissolved in 100 ml. ethanol, filtered, and mixed with 1.7 ml. diacetyl in acetic acid solution. The dichlorophenylosazone was obtained without difficulty. To confirm that the osazone and not the hydrazone had been formed, a chlorine determination was made on the dry substance. Calcd.: Cl, 35.1. Found: Cl, 34.9. Recrystallization from absolute dioxane gave macroscopic needles, m.p. 252–254°.

*Anal.* Calcd.: N, 13.8. Found: N, 13.7.

*Sorbose 2,5-dichlorophenylosazone.* 1.8 g. sorbose in a little water was heated with a filtered solution of 5.4 g. 2,5-dichlorophenylhydrazine in 54 ml. ethanol and 90 ml. 25% acetic acid. The clear, pale yellow solution was concentrated on the water-bath and hot water added gradually to avoid oil formation as the alcohol evaporated off. The first crystals precipitated after about 2 hr. and increased rapidly to give excellent yields after 6 hr. on the water-bath and 12 hr. in the incubator at 37°. After washing with dilute acetic acid, water, and CS<sub>2</sub>, and drying in the desiccator, the osazone was recrystallized from dilute alcohol. Too much water must be avoided or gels will form. Adding just enough water to cause a slight turbidity and then filtering gave long needles. The substance dissolved easily in alcohol, acetone, pyridine, carbon tetrachloride, benzol, and ethyl acetate, but tended to precipitate gelatinously on addition of petroleum ether or water to most of these solvents. It also comes out gelatinously from benzol and CCl<sub>4</sub>. Melting point 187–188°.

*Anal.* Calcd.: N, 11.3. Found: N, 11.4.

$[\alpha]_D = +15^\circ$  in the pyridine-alcohol mixture. The osazone can be regenerated by adding acetic acid to the pyridine-alcohol mixture. The flocculent yellow amorphous precipitate is slowly transformed into small needles.

*Cellobiose 2,5-dichlorophenylosazone.* 5.2 g. cellobiose, dissolved in water, was added to a clear solution of 8 g. 2,5-dichlorophenylhydrazine in 80 ml. methanol and 120 ml. 25% acetic acid. After heating on the water-bath for an hour, part of the alcohol having been allowed to evaporate, small amounts of oily or resinous precipitate were filtered off and the remainder crystallized in glittering crystals. Some bisulfite was added and the reaction completed in the incubator at  $37^\circ$ . The osazone was filtered off after 36 hr., but heating of the mother liquor was continued and a further crop obtained. The combined products gave a yield of about 62%, 50% of which was obtained in the first precipitate. Recrystallization from aqueous dioxane gave starlike needle clusters, decomposing at  $211-214^\circ$ .

*Anal.* Calcd.: N, 8.5. Found: N, 8.8.

$[\alpha]_D = +30^\circ$  in the pyridine-alcohol mixture. It can be reprecipitated with diluted acetic acid like the sorbosazone.

*Other 2,5-dichlorophenylosazones.* Reaction of xylose, arabinose, fructose, galactose, maltose, lactose, melibiose, and turanose with 2,5-dichlorophenylhydrazine in an exactly analogous way yielded the corresponding osazones. Since they were not used in this study their further investigation has been postponed.

## 2. Preparation of Phenyl Osazones

*Glycerosazone and DL-erythrosazone.* These were prepared from the corresponding alcohols, glycerol and erythritol, by oxidation with hypobromite exactly as described for the preparation of *p*-bromoglycerosazone and *p*-bromoerythrosazone. Ordinary phenylhydrazine acetate was added directly to the solution instead of the *p*-bromo derivative.

*D-Xylosazone, D-glucosazone, L-sorbosazone, D-galactosazone, maltosazone and lactosazone.* These were prepared by established procedures. *L-Arabinosazone* was made according to Hudson's (30) directions in methyl cellosolve solution.

These osazones were applied to determine the minimum amounts that can be detectably transosazonized, to demonstrate the start of the reaction, and to elucidate the reaction mechanism (see *E, F*).

## 3. Preparation of 2,4-Dinitro and *p*-Nitro Phenyl Osazones

For purposes of comparison with the products obtained by transosazonation the following nitro substituted osazones were made:

*Glyoxal and diacetyl-bis-2,4-dinitrophenylhydrazones.* These were made by heating glyoxal or diacetyl with excess 2,4-dinitrophenylhydrazine in 2 *N* HCl for 2-3 hr. on a water-bath. The products were recrystallized from nitrobenzene.

*D-Glucose, L-sorbose, D-galactose and D-glycero-D-guloaldoheptose 2,4-dinitrophenylosazones.* These were prepared according to the directions of Neuberg and Strauss (27).

*Methylglyoxal and DL-erythrose p-nitrophenylosazones.* These were prepared from the oxidation products of propylene glycol and erythritol, respectively, as described in the preparation of the corresponding *p*-bromophenylosazones. *p*-Nitrophenylhydrazine in

acetic acid solution was added to the solutions obtained. The erythrose *p*-nitrophenyl-osazone was recrystallized from pyridine and acetic acid.

*L-Arabinose and D-xylose p-nitrophenylosazones.* This preparation is described in detail because previous workers (31) could not obtain the product, probably because the heating was not sufficiently prolonged. One g. pentose and 3 g. *p*-nitrophenylhydrazine in 10 ml. 25% acetic acid and about 75 ml. ethanol were heated: first on the water-bath, then in the incubator. In the case of xylose, crystallization set in after 4 hr.; in the case of arabinose, after 5 hr. on addition of a drop of distilled water. The reaction was allowed to continue for 30 hr. Recrystallization from pyridine and acetic acid gave brick-red osazones. The arabinosazone decomposed at 240–241°, the xylosazone at 242–244°;

*Anal.* Calcd.: N, 20.0. Found: N, 20.6 and N, 19.6.

*Maltose and lactose p-nitrophenylosazones.* These were obtained in the usual way (16).

### B. Transosazonation Reactions

As mentioned above, transosazonations were found to be universal reactions. The experimental conditions differ, however, from case to case according to the solubility of the halogenated osazone used as

TABLE I  
Transosazonation Reactions

Sugar	Phenyl-osazone	Solvent	Phenylhydrazine	Acid	Hours heated	Amount isolated
						%
Glyceral	<i>p</i> -Br	Methyl cellosolve	2,4-Di-NO <sub>2</sub>	HCl	2–2 5	50
Glyceral	<i>p</i> -Br	Methyl cellosolve	<i>p</i> -NO <sub>2</sub>	Acetic	2 5	87
Methylglyceral	<i>p</i> -Br	Cellosolve + ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	4 5	66
Diacetyl	2,5-Di-Cl	Dioxane	2,4-Di-NO <sub>2</sub>	HCl	5	54
Glycerose	<i>p</i> -Br	Cellosolve + ethanol	2,4-Di-NO <sub>2</sub>	HCl	4	47
D-Erythrose	<i>p</i> -Br	Ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	10	61
D-Xylose	<i>p</i> -Br	Ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	5–6	65
L-Arabinose	<i>p</i> -Br	Ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	3–6	59
D-Glucose	<i>p</i> -Br	Cellosolve	2,4-Di-NO <sub>2</sub>	HCl	2	Theory
D-Glucose	—	Ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	5	Theory
L-Sorbose	2,5-Di-Cl	Hot ethanol	2,4-Di-NO <sub>2</sub>	HCl	1 5 + 36 at 37°	Theory
D-Galactose	<i>p</i> -Br	Ethanol	2,4-Di-NO <sub>2</sub>	HCl	4	Theory
α-D-Glucoheptose	<i>p</i> -Br	Cellosolve + methanol	2,4-Di-NO <sub>2</sub>	HCl	6	55
Maltose	<i>p</i> -Br	Ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	7 + 11 at 37°	67
Lactose	<i>p</i> -Br	Cellosolve + ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	10	38
Cellobiose	2,5-Di-Cl	Ethanol	<i>p</i> -NO <sub>2</sub>	Acetic	7 + 8 at 37°	Poor

substrate. To save space and avoid needless repetition, only two typical examples will be described and a summary of the conditions given in Table I. In all cases a 10-fold excess of nitrophenylhydrazine was used; in the case of 2,4-dinitrophenylhydrazine, solutions of the reagent in 60 ml. 2 N HCl/g. were used; *p*-nitrophenylhydrazine was applied in 25% acetic acid solution. After the requisite period of refluxing, the nitrophenylosazones were filtered off by suction and washed: first with

hot 2 *N* HCl (resp. 10% acetic acid) to remove excess phenylhydrazine which often tenaciously adheres to the product simulating unwarrantedly high yields, then with hot water until (in the case of 2,4-dinitrophenylosazones) the mother liquor was free of halogen derived from HCl. Tests for organically bound halogen were then carried out. If the product still contained traces of the halogen, these traces were removed by recrystallization. It has not been decided whether the admixture consisted of traces of the original osazone or of traces of mixed osazone.

*Glyoxal bis-p-bromophenylhydrazine to glyoxal bis-2,4-dinitrophenylhydrazine.* Twenty g. glyoxal *p*-bromophenylosazone was dissolved in 4 ml. methyl cellosolve, and a filtered solution of 2 g. 2,4-dinitrophenylhydrazine in 120 ml. 2 *N* HCl was added. When refluxed on the water-bath, the color changed to a reddish brown after 15 min. but heating had to be continued 2–2.5 hr. to obtain a pure product. (Onset of the reaction is much faster in the case of the higher sugars.) After filtering off by suction, the dinitro osazone was washed: first with hot 2 *N* HCl to remove excess reagent (hydrazine), then with hot water until the washing liquor was free from halogen. The product was then tested for organically bound halogen derived from the original *p*-bromophenylosazone.  $H_2O_2$  was used to liberate the halogen (see below, *D*). In this case small traces of halogen were still present, but these were removed by recrystallization from pyridine and glacial acetic acid, followed by washing with acetic acid and then with ether. The yield of pure isolated product was about 50%.

*Anal.* Calcd.: N, 26.8. Found: N, 26.1.

*Glyoxal bis-p-bromohydrazine to glyoxal bis-p-nitrohydrazine.* 0.2 g. glyoxal *p*-bromophenylosazone was dissolved in 4 ml. methyl cellosolve as in the previous experiment and a solution of 1.6 g. *p*-nitrophenylhydrazine in 4 ml. glacial acetic acid and 100 ml. water was added. To obtain a clear solution, an additional 45 ml. methyl cellosolve was added. After refluxing for 2.5 hr. on the water-bath, the product was filtered off by suction, washed with 10% acetic acid and then water, and tested for organically bound halogen as before. Traces present were removed by recrystallization from pyridine and toluol, and washing with toluol. Yield: 87%.

*Anal.* Calcd.: N, 25.6. Found: N, 24.9.

### *C. Colorimetric Determination*

For the colorimetric determination of the presence of 2,4-dinitro- or *p*-nitrophenylosazones the procedure of Neuberg and Strauss (17) was followed.

### *D. Halogen Determination*

Halogen was tested for according to the method of Mandel and Neuberg (19). To assure the applicability of the procedure to the substances under investigation a control experiment on an osazone of known halogen content was made. For qualitative experiments to test the absence of halogen in the transosazonation products large test tubes were used. A possible source of errors was the occasional separation of

yellow particles. These flocks were not silver halides but could be brought into solution by addition of alcohol. They may have been due to small amounts of nitrated substances not completely decomposed by the hydrogen peroxide.

### *E. Formation of Osones and Determination of the Reaction Mechanism*

1. *In the case of a pentosazone.* 1.64 g. D-xylose phenylosazone was dissolved in 130 ml. 25% acetic acid and 70 ml. water to bring the volume up to 200 ml. Since a brownish coloration began to appear after about half an hour, heating on the water-bath was not prolonged beyond 1 hr. Then the reaction mixture was placed in the refrigerator. Small quantities of resinous solids were filtered off and recrystallized from methanol, decolorizing with charcoal. They were found to yield yellow needles of typical unchanged D-xylosazone. The main part was clarified by centrifugation with bentonite or kaolin and then decolorized with charcoal to assure the adsorption of any unchanged osazone. An absolutely water-clear and colorless solution was obtained. To allow correct calculations, taking into account that some liquid is retained by the adsorbents, aliquots were used. Thus the 200 ml. originally employed gave 184 ml. after centrifuging with kaolin. This solution was made up to 250 ml. before heating with animal charcoal and yielded 210 ml. of filtrate. Two hundred ml. of this was used for the next step, the remainder being tested as follows: Fehling's solution was reduced. The naphthoresorcinol test<sup>5</sup> was positive, i.e., a violet coloration was obtained on shaking with ether. The orcinol reaction<sup>6</sup> was positive (green with amyl alcohol). The furfural test with aniline acetate was negative. A solution of *p*-nitrophenylhydrazine in 25% acetic acid immediately produced an osazone which could be identified with  $\text{NaOC}_2\text{H}_5$ . These reactions qualitatively proved the presence of osone. The exact amount formed was determined as follows: A clear solution of 1.5 g. 2,4-dinitrophenylhydrazine in 90 ml. 2 N HCl was added to the 200 ml. obtained as above. Precipitation started at once, in the cold. To complete the reaction, heating on the water-bath was continued for 2-3 hr. and the mixture placed in the incubator. To be sure that the reagent would not contaminate the osazone formed, the solution was heated again before filtering off by suction (hot), and washed thoroughly with dilute HCl and then with water. The yield of 2,4-dinitrophenylosazone obtained was 0.1435 g., corresponding to 7% osone formed.

2. *In the case of a hexosazone.* 1.8 g. D-glucose phenylosazone was dissolved in 130 ml. 25% acetic acid and 40 ml. ethanol and made up to a volume of 200 ml. with 30 ml. water. After heating on the water-bath for 3 hr., the solution was clarified by centrifuging with kaolin and then decolorized by heating with charcoal. The absolutely colorless clear solution obtained was free from unchanged osazone. An aliquot treated with a solution of 1.5 g. 2,4-dinitrophenylhydrazine in 90 ml. 2 N HCl exactly as in the case of xylose, described above, yielded after 2 hr. heating, washing, etc., 0.082 g., corresponding to 4% osone formed. (The 200 ml. originally used after evaporation of some of the alcohol and addition of water before centrifugation equalled 218 ml. These produced a clear solution of 200 ml., and with the charcoal added a volume of 220 ml. The clear filtrate ultimately obtained amounted to 185 ml., of which 175 ml. was used for the formation of the dinitrophenylosazone. The remaining 10 ml. was tested as in the case of xylosone and found to reduce Fehling's solution, give a positive

<sup>5</sup> This reaction will be fully described in a separate communication.

naphthoresorcinol and a positive orcinol test, and react with *p*-nitrophenylhydrazine acetate with formation of an osazone. While precipitation of dinitrophenylosazone commenced at once, addition of water was necessary before *p*-nitrophenylosazone formation could be detected with  $\text{NaOC}_2\text{H}_5$ .)

These experiments clearly indicate that osone is formed.

#### *F. Determination of Minimum Amounts Detectably Transosazonized*

One-tenth g. xylosazone was dissolved in methanol and water and the volume made up to 100 ml. Ten ml. of the solution so obtained was diluted to 100 ml., 1 ml. of this solution now containing 0.0001 g. or 100  $\mu\text{g}$ . 1 mg. 2,4-dinitrophenylhydrazine in 2 *N* HCl was added and heated on the water-bath for 2 hr. Visible flocks of dinitrophenylosazone formed, and the color reaction with  $\text{NaOC}_2\text{H}_5$  was clearly observable. Further dilution of 10 ml. of the previously obtained solution to 100 ml. yielded 1 ml. containing 10  $\mu\text{g}$ . A solution of 0.0001 g. dinitrophenylhydrazine in 2 *N* HCl was added to this and adsorbed on kaolin after 2 hr. heating. Centrifuging and washing with HCl followed by elution with  $\text{NaOC}_2\text{H}_5$  in the usual way (17) gave a definite violet color reaction. Further 10-fold dilution yielded 1 ml. now containing 1  $\mu\text{g}$ .; the transosazonation of this, too, could be clearly detected by absorption on kaolin and elution with  $\text{NaOC}_2\text{H}_5$ . The same series of experiments was repeated for solutions of 10  $\mu\text{g}$ . and 1  $\mu\text{g}$ . glucosazone, and 10  $\mu\text{g}$ . and 1  $\mu\text{g}$ . sorbosazone, transosazonized with 2,4-dinitrophenylhydrazine. The nitrophenylosazones formed were definitely detectable after 2 hr. heating. In the case of galactosazone, longer heating was necessary, but after 4 hr. the transosazonation of 10  $\mu\text{g}$ . and also of 1  $\mu\text{g}$ . could be clearly established. The same was found true in the case of solutions containing 10  $\mu\text{g}$ . and also 1  $\mu\text{g}$ . erythrosazone. In all these cases, the ordinary phenylosazones of the sugars were transosazonized with 2,4-dinitrophenylhydrazine in 2 *N* HCl, except in the case of erythrosazone where *p*-nitrophenylhydrazine in acetic acid was used.

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#### SUMMARY

The transosazonation reaction, *i.e.*, the exchange of the hydrazine residues in osazones, was investigated. A complete series of osazones derived from biose, triose, a tetrose, 2 pentoses, 3 hexoses, 1 heptose and 3 disaccharides as well as 2 related osazones (methylglyoxal and diacetyl bisphenylhydrazones) were studied. Conditions for the complete transosazonation of each of these were worked out.

Halogenated phenylosazones were used as starting materials; the completion of the reaction was proved by the absence of halogen in the

final product. For this purpose several osazones were prepared for the first time: diacetyl 2,5-dichloro-, sorbose 2,5-dichloro-, cellobiose 2,5-dichloro-, glyoxal *p*-bromo-, methylglyoxal *p*-bromo-, DL-erythrose *p*-bromo-, D-galactose *p*-bromo, and D-glycero-D-guloaldoheptose *p*-bromophenylosazones. Maltose and lactose *p*-bromophenylosazones were prepared for the first time by the direct interaction of the sugars with the *p*-bromophenylhydrazine. Glycero-*p*-bromophenylosazone was prepared from oxidized glycerol instead of pure glyceraldehyde as previously described.

Nitrated hydrazines were used as transosazonizing agents because they were found to yield the most insoluble products, and the osazones formed could be detected within microgram limits by a simple colorimetric test. Whenever possible, transosazonation was conducted with 2,4-dinitrophenylhydrazine in hydrochloric acid solution. In the case of erythrosazone, pentosazones, and disaccharide osazones which might be decomposed by HCl, *p*-nitrophenylhydrazine in acetic acid solution was used. The reaction was found to be a general one for the entire sugar series and related osazones.

The correct mechanism was shown to involve hydrolysis of the starting material to osones which in turn react with the nitrated phenylhydrazines to yield the less soluble nitrophenylosazones.

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# Effect of Diet on Amino Acids in Blood and Urine of Mice of Various Ages <sup>1</sup>

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## INTRODUCTION

The urinary excretion of amino acids by mice depends upon the quantity and quality of the protein ingested (1-3). It has also been noted that young thiamine-deficient mice excreted relatively high amounts of the amino acids, although the excretion by adult mice losing weight due to this deficiency was more nearly normal (4). In the present experiments, the concentrations of amino acids were determined in the plasma of mice fed certain diets that influence amino acid excretion; studies were also made of the effect of age upon amino acid excretion by mice fed diets in which the quantity or quality of the protein was abnormal.

## EXPERIMENTAL

Mice of the brown C3H, black C57, or albino strain from our own stock colony were used in this experiment. The mice were grouped according to weight, age, and sex and then were placed on the following diets:

- a) Ordinary casein at 10% of the diet,
- b) Ordinary casein at 50% of the diet,
- c) Oxidized casein (5) at 10% of the diet supplemented with DL-methionine at 0.7% of the ration (tryptophan deficient),
- d) Oxidized casein at 10% of the diet supplemented with DL-tryptophan at 0.4% of the ration (methionine deficient),
- e) Oxidized casein at 10% of the diet supplemented with DL-methionine at 0.7% and DL-tryptophan at 0.4% of the ration (completely supplemented),
- f) A ration devoid of protein, and,
- g) Ration (a) restricted to the amount eaten by group (c).

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<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The other components of the diets were corn oil 5%, salts (6) 4%, vitamins,<sup>2</sup> and glucose monohydrate ("Cerelese") to 100%. These are essentially the diets used in previous studies from this laboratory (1,2).

In the first series, the mice averaged 12–14 weeks in age. In a second series, weanling mice 21–28 days in age were placed on diets (a), (b), and (c). The mice were kept on all diets at least 2 weeks before samples of blood or urine were collected. Losses in weight ranged from 20 to 34% for the animals fed diets deficient in tryptophan or methionine, calories, or protein. The mice on the completely supplemented oxidized casein [diet (e)] lost only 9% of their weight. The weanling mice fed ordinary casein [diets (a) and (b)] gained in weight, and the older mice on these diets maintained their weights.

In studies of the effect of age on excretion, groups of weanling mice were fed the appropriate diets and were placed in metabolism cages during the 5th and 13th weeks of life. One group of 12 was fed the control diet (10% of casein) for 14 weeks, and 3-day collections of urine were taken at biweekly intervals. The analyses reported were made on pooled 3-day samples from groups of 3–6 mice each.

Blood samples were procured by lightly anaesthetizing the animals with ether, after which the thoracic cavity was opened and the blood was withdrawn directly from the heart into a syringe rinsed with a 3% sodium citrate solution, and then placed into centrifuge tubes containing 30 mg. of dry sodium citrate. After centrifugation the plasma was removed and deproteinized with tungstic acid according to the method of Hier and Bergeim (7). The blood from at least 10 mice was pooled for each sample and hematocrit values were determined on the pooled samples. The methods used to collect urine (1), to prepare the samples for assay (2), and to determine the specific amino acids microbiologically (1,8) have been described previously. For the determination of the amino acids in deproteinized plasma, a micro method was used in which the final volume/tube was 0.4 ml. (9). The  $\alpha$ -amino nitrogen content of the urines was determined by the Cu method of Clayton *et al.* (10).

## RESULTS

### *Plasma Amino Acids*

When adult mice were deprived of protein for 1–2 weeks, the concentrations of 3 representative amino acids: tryptophan, phenylalanine,

<sup>2</sup>  $\mu\text{g./g.}$  of ration:

Pyridoxine hydrochloride	6
Thiamine chloride	6
Nicotinic acid	10
Calcium pantothenate	20
Riboflavin	6
Folic acid	0.5
Biotin	0.5
<i>p</i> -Aminobenzoic acid	300
Inositol	500
Choline chloride	1000
(Cystine)	1000)

and valine, dropped to less than half of those found when a ration containing 10% of casein was fed (Table I). Isoleucine, however, was present in relatively high amounts in the deficient plasma ( $\frac{2}{3}$  of normal) and fell within the range sometimes encountered in normal individuals. The decreases in amino acid concentration were not accompanied by any alteration in the plasma-cell ratio of the blood; the hematocrit values in the "no protein" group ranged from 35 to 39%, while the hematocrit values of the control mice averaged 38%.<sup>3</sup>

TABLE I  
*Representative Amino Acids in Plasma of Adult Mice Fed a Protein-Free Diet or One Containing 10% of Casein*

Diet	10% of casein $\mu\text{g} / \text{ml}$	No protein $\mu\text{g} / \text{ml}$
Tryptophan	12	2
Phenylalanine	23	7
Valine	41	19
Leucine	22	12
Isoleucine	12	8

Table II lists the amounts of 15 amino acids found in the pooled blood of adult mice fed diets known to alter amino acid excretion. Aspartic acid and cystine were not included since they were present in the plasma in only trace amounts; serine was omitted from the table because the B<sub>6</sub> vitamins interfere with the assay. In general the various diets did not exert much effect on the concentration of the amino acids in the plasma. Essentially similar concentrations were found whether the diet contained 10 or 50% of casein; alanine, isoleucine, leucine, lysine, phenylalanine, and valine increased slightly when the high-protein diet was fed, but these increases were not significant. When adult mice were deprived of tryptophan or methionine, or restricted in food intake, the amounts of the individual amino acids in the blood were very similar to those in the control groups. However, the plasma of mice on diets deficient in tryptophan contained relatively little tryptophan while that of mice fed the methionine-deficient ration contained relatively little methionine (Table II).

Urines collected from these groups were analyzed for 4 representative amino acids, leucine, valine, histidine, and alanine, with the following

<sup>3</sup> These represent values for blood diluted with the sodium citrate solution in the syringe and do not represent true hematocrit values for the mouse.

mean percentages of excretion (mg. excreted  $\times$  100/mg. ingested):

- 4.6% on ordinary casein;
- 3.9% on oxidized casein supplemented with both tryptophan and methionine;
- 10.7% on the low tryptophan diet;
- 10.0% on the low methionine diet;
- 3.4% when ordinary casein was fed, but with the food intake restricted to that consumed by the mice on the tryptophan-deficient diet.

These differences in amino acid excretion were evident in spite of the relative constancy of the levels in the blood. In weanling mice, also,

TABLE II

*Amino Acids in the Plasma of Adult Mice Fed Various Diets*  
(All values in  $\mu\text{g./ml. of plasma}$ )

Diets	a 10% casein	b 50% casein	c Tryptophan deficient	d Methionine deficient	e Completely supple- mented	f Restricted
Alanine	53	57	61	81	66	55
Arginine	9	10	8	10	10	5
Glutamic acid	35	29	40	43	36	43
Glycine	23	17	14	16	17	13
Histidine	17	16	11	14	14	13
Isoleucine	12	20	8	14	12	21
Leucine	22	28	18	20	22	18
Lysine	57	61	49	51	70	48
Methionine	17	18	23	12	22	19
Phenylalanine	23	29	18	18	20	22
Proline	18	16	18	23	21	18
Threonine	39	36	28	44	30	24
Tryptophan	12	11	5	11	14	14
Tyrosine	25	27	18	21	24	21
Valine	41	50	29	34	38	42

the concentrations of the amino acids in the blood were very similar whether the diet fed contained ordinary casein or a treated casein low in tryptophan (9).

*Effect of Age on Variations in Urinary Amino Acids  
With a Change in Diet*

Weanling mice fed a diet containing 10% of casein excreted essentially the same amounts of 4 representative amino acids at 4 weeks of age as at 14 weeks of age (Table III), and the percentages of the various ingested amino acids appearing in the urine in the "free" form did not appear to vary with the age of the animal. "Total" amino acids as

TABLE III  
*Excretion of Four Amino Acids by Control Mice of Various Ages  
Fed a 10% of Casein Diet*

Age	Weeks					
	4	6	8	10	12	14
Leucine						
intake, mg./day	28.6	28.8	29.2	26.6	25.6	26.8
mg. "free" leucine excr./day	0.63	0.56	0.86	0.77	0.57	0.80
% excreted	2.2	1.9	3.0	2.9	2.2	3.0
mg. "total" leucine excr./day	0.86	1.22	1.33	1.30	1.20	1.37
% excreted	3.0	4.2	4.6	4.9	4.7	5.1
Valine						
intake, mg./day	17.6	17.6	17.8	16.2	15.6	15.2
mg. "free" valine excr./day	0.64	0.97	0.75	0.61	0.79	0.78
% excreted	3.6	5.5	4.2	3.8	5.1	5.1
mg. "total" valine excr./day	0.91	1.20	1.05	0.91	1.09	1.12
% excreted	5.2	6.8	5.9	5.6	7.0	7.4
Histidine						
intake, mg./day	7.2	8.3	8.4	7.6	7.3	7.1
mg. "free" histidine excr./day	0.22	0.29	0.22	0.22	0.23	0.22
% excreted	3.1	3.5	2.6	2.9	3.2	3.1
mg. "total" histidine excr./day	0.27	0.36	0.42	0.39	0.43	0.45
% excreted	3.8	4.2	5.0	5.1	5.9	6.4
Alanine						
intake, mg./day	9.2	9.4	8.2	8.7	8.3	8.1
mg. "free" alanine excr./day	0.44	0.54	0.42	0.48	0.54	0.49
% excreted	4.8	5.8	5.1	5.5	6.5	6.1
mg. "total" alanine excr./day	0.53	0.75	0.68	0.69	0.75	0.71
% excreted	5.8	8.0	8.3	7.9	9.0	8.8

TABLE IV

*Excretion of Four Amino Acids by Young and Adult Mice Fed High Protein  
or a Diet Deficient in Tryptophan*

Diet.... .	50% of casein		Tryptophan-deficient diet	
	4 wks.	12 wks.	4 wks.	12 wks.
Age of mice.....				
Leucine				
intake, mg./day	156	153	14.1	28.5
mg. "free" leucine excr./day	3.5	1.3	1.4	0.9
% excreted	2.2	0.8	10.0	3.1
mg. "total" leucine excr./day	4.4	3.5	2.0	1.4
% excreted	2.8	2.3	14.2	4.8
Valine				
intake, mg./day	95	93	8.3	17.4
mg. "free" valine excr./day	3.2	1.5	1.5	0.8
% excreted	3.4	1.7	18.0	4.8
mg. "total" valine excr./day	4.1	2.5	2.0	1.1
% excreted	4.3	2.7	23.4	6.1
Histidine				
intake, mg./day	44.8	43.6	3.2	6.6
mg. "free" histidine excr./day	1.4	0.6	0.7	0.3
% excreted	3.1	1.4	20.1	4.9
mg. "total" histidine excr./day	1.5	1.2	0.7	0.4
% excreted	3.4	2.7	20.1	5.3
Alanine				
intake, mg./day	50.7	47.1	4.5	9.2
mg. "free" alanine excr./day	1.7	1.2	1.0	0.5
% excreted	3.3	2.5	22.0	5.4
mg. "total" alanine excr./day	2.3	1.7	1.0	0.7
% excreted	4.5	3.6	22.0	7.4

measured in acid-hydrolyzed urine were excreted in somewhat greater amounts as the age of the mice increased, *e.g.*, mice 4 weeks of age excreted a mean percentage of 4.5 of the ingested amino acids, while the older animals excreted a mean percentage of 6.9.

However, when the quality or quantity of dietary protein was altered the responses of the mice as measured by the excretion of leucine, valine, histidine, and alanine depended significantly on the age of the animals. The older mice fed 50% of casein excreted more of each of the amino

acids than similar mice fed 10% of casein, but the magnitude of this increase was considerably less than that observed in younger animals; roughly 2.5-fold in the older animals and 4-5-fold in the young ones (Tables III and IV).

The ages of the mice appeared to exert an even greater influence on amino acid excretion when the dietary protein was devoid of tryptophan. The older mice ate approximately the same amount of the deficient diet (Table IV) as when 10% of ordinary casein was fed (Table III), and the amounts of the 4 amino acids in the urine were also practically identical on the 2 diets (Tables III and IV). On the other hand the 4-week old mice consumed only half as much food on the deficient diet as on the control diet, yet the excretion of each of the 4 amino acids in both the free and combined forms was significantly higher in the deficient group (Table IV) than by control mice of similar age (Table III). Expressed as the percentages of the amounts of the 4 ingested amino acids appearing in hydrolyzed urine, the 4-week old mice excreted 14.2-23.4% on a diet low in tryptophan (Table IV) and only 3.0-5.8% on the control diet (Table III). In other words, amino acid excretion by young mice was markedly increased when an incomplete protein was fed, although the excretion of the amino acids by the older animals was much less sensitive to this type of deficiency.

## DISCUSSION

The present results emphasize the fact that appropriate dietary changes may cause significant alterations in the amounts of the amino acids in mouse urine without any comparable changes in the amino acid content of the blood. Amino acids are therefore similar to other substances in that the amounts in the blood are relatively constant as compared with the amounts in the urine. Only in mice fed a diet devoid of protein was any significant deviation noted from the amounts of the amino acids found in normal plasma. Henderson *et al.* (11) also noted a reduction of plasma amino acids in rats fed a protein-free ration as compared to those on a diet containing 18% of casein.

Young mice excreted excess amino acids when fed dietary protein that was abnormal either in quantity or quality. In contrast, adult mice on a tryptophan-deficient diet did not excrete particularly high amounts of the amino acids, and on a diet containing 50% of casein the increase in excretion was comparatively moderate. However, this

difference between young and older animals disappeared when the diet contained 10% of ordinary casein, and the older animals actually excreted higher amounts of bound amino acids than did the weanlings. Presumably when the amino acids in the blood exceed a normal level, they may be incorporated into tissue protein, they may be destroyed by transamination or other reactions, or they may pass into the kidney tubules and fail to be reabsorbed. Since adult mice fed abnormal protein revealed no unusual accumulation of amino acids in either the plasma or urine in spite of a loss in body weight, it may be concluded that the physiological processes for destroying amino acids were unimpaired in these animals. In the young mouse, on the other hand, the mechanisms for the reabsorption of the amino acids and for their destruction appeared to be adequate when the dietary protein was balanced in quality and moderate in amount, but at least one of these mechanisms was inadequate to cope with the amino acids in a high-protein diet or those in one containing an incomplete protein, and the final result was a loss of amino acids in the urine.

### SUMMARY

1. The concentrations of amino acids in mouse blood were essentially the same whether the diet fed contained 10% or 50% of casein, although plasma from mice on a protein-free diet contained relatively low amounts of the amino acids. Other diets known to alter amino acid excretion (diets low in tryptophan or methionine, or a ration restricted in calories) did not produce any marked change in the amino acid content of the blood.

2. When the diet contained 10% of ordinary casein, mice 4-14 weeks of age excreted approximately similar amounts of amino acids in the urine. When the dietary protein was abnormal either in quantity or quality, young mice excreted much higher percentages of the ingested amino acids in the urines than older mice.

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# Preparation and Properties of a Purified Antisecretory Substance: Urogastrone

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## INTRODUCTION

As a result of clinical observations indicating that pregnancy has a beneficial effect on symptoms of peptic ulcer, Sandweiss, Saltzstein and Farbman (1) studied extracts of pregnancy urine and found them to have prophylactic and therapeutic effects on experimental Mann-Williamson ulcers. Stimulated by this lead, many workers have studied the effects of urine extractives on experimental ulcers, human peptic ulcers, and gastric secretion. From these studies have come the concepts of (a) anhelone, an ulcer inhibiting substance in urine; (b) urogastrone, a gastric secretory inhibitory substance in urine; and (c) the anhelone or urogastrone activity of human chorionic gonadotrophin (2). The postulated substance, anhelone, has resisted all efforts at separation and purification. Its existence is inferred from indirect biological observations. Several procedures for the extraction and purification of urogastrone have been described, but it does not appear likely that any major increase in biological activity has been accomplished in the 10 years since Gray and co-workers (3) first described a method for preparing this substance. The urogastrone activity of chorionic gonadotrophin has been studied extensively, but to date impure preparations have been used so that it has been impossible to relate absolutely urogastrone and chorionic gonadotrophin.

This report concerns an attempt to purify urogastrone so as to study some of its properties and also to describe a protein of very high purity that possesses the biological activity of urogastrone.

## EXPERIMENTAL

Three techniques have been employed for the measurement of biological activity. First, anti-ulcer activity has been studied in the Shay rat (4) as modified by this

laboratory (5). Second, we have used the well-known acute experiment in which gastric secretion in a total stomach pouch of the anesthetized dog is stimulated by continual injections of histamine and the secreted juice is collected from a cannula inserted into the stomach. Third, the gastric secretion assay in rats as first described by Friedman and co-workers (6) and as modified by this laboratory (5) has been used. This latter method appears to give the most quantitative index of activity of preparations in inhibiting gastric secretion of any of the methods that have been employed in this laboratory. Test substances are administered at three dosage levels and the log dose-response relationship is established graphically. This relationship is linear within certain dosage limits and the interpolated dosage giving a 50% inhibition of secretion is used as an index of activity.

### *Isolation Procedure*

A schematic diagram showing the fractionation procedure that has been employed is presented in Table I. The first step, which is adsorption on benzoic acid, is common to most of the procedures that have been described for the preparation of urogastrone and is the usual first step involved in procedures for the isolation of protein substances such as chorionic gonadotrophin from urine. On exhaustive dialysis of the water extract of the benzoic acid adsorbed material, about 20% of the total solids and essentially all of the activity is recovered in the nondialyzable fraction. When the nondialyzed material is adjusted to pH 4.3, about 50% of the solids are removed as an inactive protein precipitate. The filtrate from this isoelectric precipitation is digested with trypsin, and dialyzed. The nondialyzable fraction yields approximately 800 mg. of active urogastrone from the original 100 l. of urine.

This final fraction was obtained as a gray, nonhygroscopic amorphous solid. It was quite soluble in water, sparingly soluble in dilute alcohol, and insoluble in organic solvents that are immiscible with water. The purified preparation contained about 3.5% ash and 9.5% nitrogen and showed positive qualitative tests for sulfur, carbohydrate (Molisch) and protein. It did not contain phosphorus.

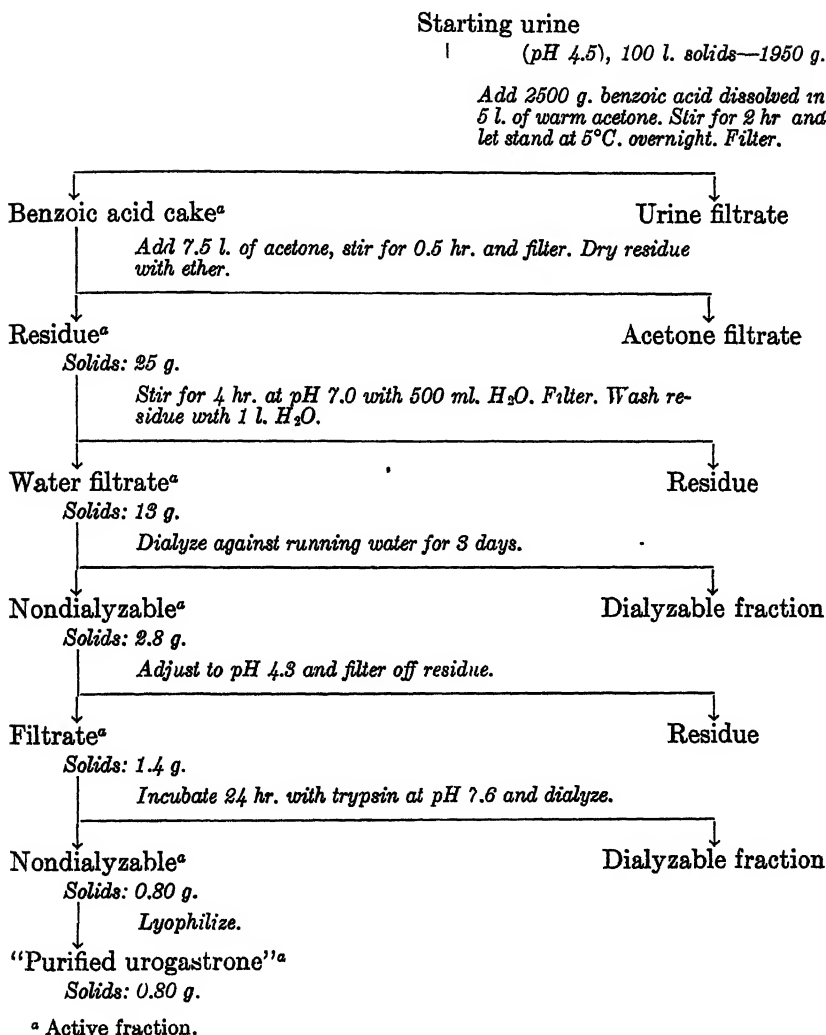
The purified urogastrone obtained by this method probably is not a single substance and the determination of its exact chemical nature must await further purification. The properties of this preparation indicate that urogastrone is a large nondialyzable molecule, probably containing carbohydrate and protein or polypeptides; very likely it is a glycoprotein.

### *Biological Activity*

In the Shay rat, 25 mg./rat when administered intraperitoneally at the time of ligation of the pylorus completely prevented the formation of gastric ulcers. This is an activity considerably greater than that of a urogastrone preparation made by a procedure described by Gray *et al.* (7), and reported earlier from these laboratories to suppress almost completely ulceration at a dosage of 50 mg./rat (5).

The same urogastrone preparation made by the procedure of Gray *et al.* also was reported to inhibit almost completely gastric secretion in the 5-hour rat assay at a dosage of 5 mg./rat (approximately 70% inhibition). This would appear to be of the

TABLE I  
The Preparation of Purified Urogastrone from Urine



same order of activity as the purified preparation that is described in the present report (see Fig. 1).

In the total stomach pouch dog, the purified urogastrone caused a 74% inhibition of histamine-stimulated gastric secretion at a dosage of 0.5 mg./kg. (see Fig. 1).

Comparing this result with the published report of Gray and co-workers (7), it would appear likely that in the dog the activity of the present urogastrone is approximately the same as the earlier preparation.

The inherent errors of the gastric secretion assay procedures are large. Undoubtedly errors of at least  $\pm 25$ -50% can be expected. The anti-ulcer assay in the Shay rat has been more variable than other procedures and it is probable that the apparent lack of correlation with the gastric secretion assays is due to the assay method rather than to a specific effect on the ulceration process as has been postulated for the substance, anthelone.

It is of some interest to compare the biological activity of urogastrone in the rat and dog with that of enterogastrone. The urogastrone described in this report was compared with enterogastrone that had been prepared from fresh hog intestines by

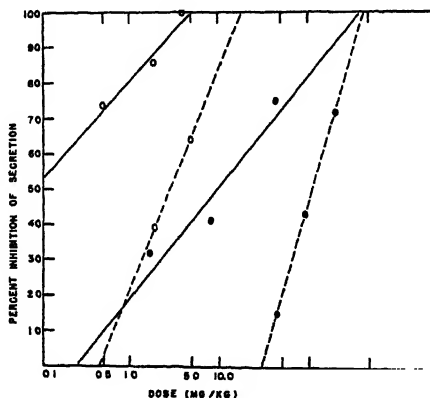


FIG. 1. Log dose-response relationship of urogastrone and enterogastrone. ○—○, urogastrone by the dog assay; ○---○, enterogastrone by the dog assay; ●—●, urogastrone by the rat assay; ●---●, enterogastrone by the rat assay.

the picric acid method of Greengard *et al.* (8). Inhibition of secretion was measured by the rat and dog methods that were mentioned previously. The log dose-response relationship is shown graphically in Fig. 1. Perhaps the most striking thing that is illustrated here is the difference in slope that is obtained from enterogastrone and urogastrone. This difference is typical, having been observed consistently with different preparations and emphasizes a difference in biological activity that has not been pointed out previously. It is apparent that a quantitative comparison of activity of these two preparations cannot be made with either of the assay procedures. On the other hand there is a general parallelism between responses in the rat and the dog for each of the preparations. When referred to body weight much larger doses of the two preparations are required for a given inhibition of secretion in the rat assay than in the dog assay.

*Urogastrone Activity of Human Chorionic Gonadotrophin*

It has been found by many workers that crude preparations of chorionic gonadotrophin give typical urogastrone responses. Since standard procedures for the preparation of chorionic gonadotrophin are very similar to the procedures employed in the isolation of urogastrone, it was of considerable interest to determine whether the urogastrone activity was due to the chorionic gonadotrophin per se or to an impurity that was present in crude preparations. Gurin, Bachman and Wilson (9) have described in detail the preparation and evidence of purity of chorionic gonadotrophin from human pregnancy urine. Three preparations of chorionic gonadotrophin were kindly supplied by Dr. Gurin for the urogastrone studies presented here. These preparations represented three stages of purity of the material, the purest being a noncrystalline homogeneous preparation as determined by electrophoretic and ultracentrifugal methods. When assayed for inhibition of gastric secretion in the rat, these preparations showed marked inhibition of secretion and appeared to be approximately as active as the urogastrone described above. The results of these assays are shown in Table II.

TABLE II  
*Inhibition of Secretion by Chorionic Gonadotrophin*

Preparation	Dose/rat for 50% inhibition of secretion mg.
Urogastrone (reference control)	1.2
Prolan (1.0 $\gamma$ contains 1 rabbit unit)	1.8
Prolan (0.5 $\gamma$ contains 1 rabbit unit)	1.0
Prolan (0.25 $\gamma$ contains 1 rabbit unit)	1.5

Chorionic gonadotrophin is known to be very unstable to heat. Considerable loss of gonadotrophic activity has been caused merely by allowing preparations to stand at room temperature for as short a period as 24 hr. One of the purified gonadotrophic preparations was subjected to heat and to digestion by either trypsin or amylase. Most of the gonatrophic activity was destroyed by these drastic procedures, but, surprisingly enough, a small percentage of the original gonadotrophic activity remained in spite of the severity of the treatment. When tested for inhibition of secretion in the rat, the urogastrone activity was not lost. This is shown in Table III. From these observations it is evident that gonadotrophic activity is not responsible for inhibition of gastric secretion. Substantiating this conclusion is the fact that the urogastrone preparation described above did not exhibit any gonadotrophic activity in the rat at a level as high as 25 mg./rat. This latter observation confirms the results of Gray *et al.* (7), and of Sandweiss, Saltzstein and Farbman (10).

TABLE III  
*Inhibition of Secretion by "Inactivated" Chorionic Gonadotrophin*

Preparation	Dose/rat for 50% inhibition of secretion mg.
Prolan (control—no treatment)	1.0
Prolan (trypsin digested)	1.6
Prolan (amylase digested)	0.8

## DISCUSSION

In the urogastrone purification procedure that has been described, it appears likely that a potency approximating a maximum has been obtained and that this potency is approximately the same as that found by other investigators employing different purification procedures. It is probable that the urogastrone preparation described here is not pure. The presence of impurities accounting for as much as 25% of the preparation could be present and yet not be evidenced by a measurable change in biological activity because of the inherent variability of the assay procedures that are now available.

The early observation that peptic ulcer is affected beneficially by pregnancy and that urogastrone excretion in the urine is increased greatly during pregnancy has led to the belief that chorionic gonadotrophin may be concerned with this effect. Gonadotrophic activity per se is not necessarily associated with inhibition of gastric secretion. However, the basic structure of the molecule or an active moiety must be concerned with the urogastrone effect since gonadotrophins of very high states of purity are active in inhibiting gastric secretion.

The observation that a homogeneous chorionic gonadotrophin possesses the biological characteristics of urogastrone offers for the first time a means of settling the question of the existence of a separate entity having specific anti-ulcer properties. A reasonably simple procedure is now available for the preparation of crystalline chorionic gonadotrophin (15). It would be interesting to see whether such material possesses anthelone properties; for, such an effect, if obtained, logically could not be assigned to an impurity.

Kaulbersz *et al.* (11,12) have shown that urogastrone excretion in the urine was depressed markedly by hypophysectomy. Extirpation of the ovaries seemed to depress excretion to some extent but removal of other endocrine glands was without effect. This observation makes it appear likely that a substance very closely related to the protein hormones of the pituitary, possibly pituitary gonadotrophin—both L. S. H. and F. S. H. are known to be glycoproteins—is the parent substance of the urogastrone that is excreted in the urine of nonpregnant humans. Gray, Harris and Wiczorowski (13) have shown that the posterior pituitary hormones are not identical with urogastrone. Since urogastrone increases markedly in the urine of pregnant females, it is possible that this urogastrone is of chorionic

origin. Chemical studies of purified urogastrone do not indicate any inconsistencies with the known chemical properties of chorionic gonadotrophin. It is evident from the studies that have been reported here that urogastrone of pituitary or chorionic origin does not necessarily possess gonadotrophic activity. It may be excreted in the urine in an inactive form, or gonadotrophic activity might be destroyed in the process of extraction of the urogastrone from the urine. Hanke (14) has described a crystalline protein from normal human urine that possesses urogastrone properties. It would be interesting to compare the properties of this material with pure, heat-inactivated chorionic gonadotrophin.

### SUMMARY

1. A method for the preparation of relatively pure urogastrone from human urine has been described. The chemical properties of this preparation support the postulate that urogastrone is a glycoprotein.

2. A comparison of inhibition of gastric secretion by urogastrone and enterogastrone has shown that the log dose-response relationships of these two substances have different slopes so that quantitative comparisons of activity cannot be established.

3. The gastric secretion inhibitory properties of a homogeneous preparation of chorionic gonadotrophin are approximately the same as were obtained with purified urogastrone. This urogastrone activity was not related to gonadotrophic activity since inactivation of the gonadotrophin did not affect urogastrone activity.

4. A hypothesis relating urogastrone to urinary glycoproteins of pituitary and chorionic origin has been presented.

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# The Desulfurization of Protein Hydrolysates by Treatment with Raney Nickel <sup>1,2</sup>

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## INTRODUCTION

For studies on the metabolism of sulfur-containing compounds, we desired a basal amino acid mixture which was free of cystine and methionine. Protein hydrolysates offered an economical and readily available source of mixtures of L-amino acids. However, the composition of the mixture in an hydrolysate is dependent upon the nature of the protein and the method of preparation. Sulfur-free proteins for hydrolysis were unknown.

It seemed likely that the desulfurization reaction employing Raney nickel catalyst (1) might offer a new method for the preparation of sulfur-free mixtures of amino acids from protein hydrolysates. The reagent had been shown to convert L-cystine to L-alanine, and L-methionine to L- $\alpha$ -aminobutyric acid (2), by reductive elimination of the sulfur which was removed as nickel sulfide. We have found that protein hydrolysates likewise may be quantitatively desulfurized with Raney nickel. Presumably the desulfurization reaction produces no new organic constituents other than L-amino acids.

The method has been applied to an acid hydrolysate and to an enzymatic hydrolysate. Each hydrolysate was shaken with Raney nickel in mildly alkaline solution at room temperature. Nickel sulfide was removed with the catalyst. The major portion of the amino acids was in the filtrate. Some of the material which was adsorbed on the

<sup>1</sup> This study was supported by grants from the Nutrition Foundation, Inc., and the Rockefeller Foundation.

<sup>2</sup> A preliminary report of this study was presented at the Detroit meeting of the American Institute of Nutrition, April 19, 1949.

nickel was removed by elution processes. Effects of the treatment on the character of each hydrolysate were assayed by chemical tests, paper chromatography, and a test of its ability to support the growth of young rats.

## EXPERIMENTAL

### *Enzymatic Hydrolysate*

Twenty grams of a commercial preparation<sup>3</sup> were dissolved in 125 ml. of water, and 20 ml. of 5%  $\text{Na}_2\text{CO}_3$  were added. The pH was made alkaline to phenolphthalein with 0.1 N NaOH. Forty grams of Raney nickel catalyst<sup>4</sup> (3) were added and the mixture was shaken for 3 hr. at room temperature. A qualitative test for organic sulfur was positive. After shaking for an additional 2 hr., the hydrolysate was free of

TABLE I  
*Enzymatic Hydrolysate\**

	Untreated %	Desulfurized %
Cystine	0.22	0.00
Methionine	3.0	0.0
Total N recovered	100	67.4
Amino N/total N	45	43
Ammonia N/total N	3.5	11
Threonine N/total N	3.0	3.5
Phenylalanine N/total N	3.1	1.9

### *Acid Hydrolysate*

Cystine	0.00	0.00
Methionine	3.6	0.0
Total N recovered	100	76
Amino N/total N	54	60
Ammonia N/total N	2.6	3.7
Threonine N/total N	1.6	2.1
Phenylalanine N/total N	1.67	1.63

\* This preparation was dried according to the procedure of Westfall, Miller and Westfall (13) by adding dextrin to a syrup before lyophilization.

sulfur. The solution, still containing the catalyst, was saturated with  $\text{H}_2\text{S}$  and was allowed to stand at room temperature for some time before filtering. The filtrate contained  $\text{NiS}$  colloid which was precipitated by repeated freezing and thawing. The  $\text{NiS}$  was removed by filtering. A Kjeldahl nitrogen determination indicated 75% recovery of the nitrogen. In one case, traces of sulfate sulfur which were present were removed by precipitation with barium hydroxide. In another preparation, 67% of the nitrogen was recovered. Results of analysis of this product are shown in Table I.

<sup>3</sup> We are indebted to Dr. Warren M. Cox, Jr., of the Mead Johnson Co. for a generous supply of "Amigen."

<sup>4</sup> We are indebted to Mr. Murray Raney for Raney nickel alloy.

*Acid Protein Hydrolysate*

Seventy-five grams of freshly prepared Raney nickel catalyst was added to 60 g. of protein hydrolysate<sup>1</sup> dissolved in 400 ml. of water. The pH of the solution was adjusted to 8.2 by adding 1 *N* NaOH. The mixture were shaken at room temperature for 2 hr. At this time a qualitative test for organic sulfur was positive. An additional 25 g. of catalyst were added and the solution was shaken for another hour. A test for sulfur was negative.

The solution was freed of nickel by the procedure used for the enzymatic hydrolysate.

The solution was adjusted to about pH 5 with concentrated HCl and then was aerated for 2 hr. to remove excess H<sub>2</sub>S and to precipitate free sulfur. The sulfur was removed by filtration and the volume of the filtrate reduced to 450 ml. by evaporation at 50°C. under reduced pressure. The concentrated solution was lyophilized to obtain the desulfurized product as a dry powder. Quantitative nitrogen determination by the Kjeldahl method indicated that 72% of the original nitrogen present in the hydrolysate had been recovered.

A repetition of the procedure yielded a 76% recovery of total nitrogen.

Results of chemical analyses on preparations used in the growth studies are summarized in Table I. Methionine was determined by the method of McCarthy and Sullivan (4), cystine by the method of Sullivan and Hess (5), and phenylalanine by Block and Bolling's adaptation of the Kapeller-Adler-Kuhn method (6). Threonine was determined by the method of Shinn and Nicolet (7), after the samples had been hydrolyzed for 24 hr. with 6 *N* HCl. Total nitrogen was determined by the Kjeldahl method, and amino nitrogen by formol titration using neutral formaldehyde. Ammonia nitrogen was determined by aeration into standard sulfuric acid.

Qualitative organic sulfur tests were carried out in the following manner: To a solution containing 15–20 mg. of the substance in a 30-ml. beaker were added 0.1 ml. of 20% Mg(NO<sub>3</sub>)<sub>2</sub> and 1.5 ml. of 70% HClO<sub>4</sub>. The solution was boiled on a hot plate for a few minutes while a watch glass covered the beaker. The watch glass was then removed and the solution was evaporated to dryness. The residue was dissolved in 0.5 ml. of 1% HCl, transferred to a small test tube, and treated with 2–4 drops of 5% BaCl<sub>2</sub> solution. A turbidity was noted when 8 µg. of sulfur was present.

Two-dimensional paper chromatograms, using phenol and collidine-lutidine, were run on hydrolysates before and after treatment with Raney nickel catalyst (8). The enzymatic hydrolysates were hydrolyzed with acid before being tested. After treatment of the paper with ninhydrin, the spot for methionine was missing in chromatograms of desulfurized preparations. The α-aminobutyric acid spot was correspondingly increased. The amounts of cystine in the original solutions were less than the sensitivity of the test.<sup>2</sup> The distribution of other spots was not changed by the desulfurization reactions.

<sup>1</sup> "Parenamine," a 15% solution of an acid hydrolysate of casein supplemented with 1% tryptophan.

<sup>2</sup> Cystine is not seen in a chromatogram unless large quantities are used, in which case traces of cysteic acid may be detected (8).

Young rats of the Long-Evans strain were used for the growth studies. Litter mates were placed in individual cages soon after weaning. Food and water were supplied *ad lib*. Each of the diets had the following composition: protein hydrolysate under test, 20; sucrose, 32; dextrin, 32.65; salt mixture, Jones-Foster (9), 4; corn oil, 2;

TABLE II  
*Growth Response of Young Rats to Desulfurized Protein Hydrolysates*

Enzymatic hydrolysate					Acid hydrolysate				
Rat no. (initial weight)	Days on diet	Average daily food intake	Total weight change	Nature of hydrolysate and supplement	Rat no. (initial weight)	Days on diet	Average daily food intake	Total weight change	Nature of hydrolysate and supplement
2 (43)	4	6.5	+7.5	Untreated	17 (32)	4	7.0	+1.8	Untreated
	4	5.2	-7.0	Desulfurized		8	6.2	-13.0	Desulfurized
	8	7.5	+10.5	Desulfurized plus 0.8% methionine and 0.2% cystine		12	4.1	+8.1	Desulfurized plus 0.8% methionine and 0.2% cystine
	8	5.5	-9.5	Desulfurized		4	7.0	-7.4	Desulfurized
	8	6.0	+23.3	Untreated					
3 (45)	4	6.0	+8.5	Untreated	18 (35)	4	7.2	+1.7	Untreated
	4	7.5	-5.2	Desulfurized		8	5.2	-6.8	Desulfurized
	16	9.4	+28.7	Desulfurized plus 0.8% methionine and 0.2% cystine		8	5.0	+3.7	Desulfurized plus 0.8% methionine and 0.2% cystine
	8	10.2	+22.4	Untreated					
4 (43)	4	3.0	+6.0	Untreated	19 (51)	8	4.0	+7.9	Untreated
	4	5.1	-4.0	Desulfurized		4	5.0	-7.3	Desulfurized
	16	6.7	+19.5	Desulfurized plus 0.8% methionine and 0.2% cystine		8	5.2	+3.3	Desulfurized plus 1% methionine
	8	10.2	+11.5	Untreated					
5 (36)	4	4.0	+5.5	Untreated	20 (51)	4	7.2	+6.5	Untreated
	12	5.0	-9.5	Desulfurized		8	5.6	-7.5	Desulfurized
	8	3.0	-3.0	Desulfurized plus 0.5% cystine		12	4.7	-6.2	Desulfurized plus 0.5% cystine
	8	4.0	+17.0	Untreated					
6 (44)	4	3.2	+7.5	Untreated	21 (52)	4	7.5	+3.0	Untreated
	16	4.6	-15.3	Desulfurized		16	5.1	-11.0	Desulfurized
	4	6.7	+8.5	Desulfurized plus 0.8% methionine and 0.2% cystine		4	4.0	+1.5	Desulfurized plus 0.8% methionine and 0.2% cystine
	8	4.3	+10.8	Untreated					
					22 (58)	8	6.0	+1.5	Untreated
						12	5.7	-10.4	Desulfurized
						4	4.0	+3.4	Desulfurized plus 0.8% methionine and 0.2% cystine
					23 (48)	32	7.6	+30.5	Untreated
					24 (58)	32	9.2	+43.0	Untreated
					25 (61)	16	7.7	+17.5	Untreated

lard, 7; Cellu flour, 2; cod liver oil, 0.05; choline chloride, 0.2; inositol, 1.0; alpha tocopherol 0.002. Methionine and cystine, when used as supplements, were mixed with the diet at the time of feeding. Water-soluble vitamins were added to the food just before it was placed in the cages. The following amounts were added to each kilogram of diet: thiamine hydrochloride, 5 mg.; riboflavin, 10 mg.; pyroxidine hydrochloride, 5 mg.; nicotinic acid, 5 mg.; calcium-D-pantothenate, 25 mg.; *p*-aminobenzoic acid, 300 mg.; 2-methyl-1,4-naphthoquinone, 2 mg. Weighings were made each 4 days. Growth response, measured as change in weight, is recorded in Table II.

## RESULTS AND DISCUSSION

The success of the desulfurization reaction was demonstrated by the qualitative test which showed the absence of sulfur in the product. Kjeldahl nitrogen determinations showed that from one-quarter to one-third of the nitrogenous materials of the solution remained adsorbed on the nickel-nickel sulfide residues after the elution processes. This loss required a study of the constituents left in the solution. The adsorption process had possibly altered the relative quantities of individual amino acids in the mixture. Tests showed, however, that the alteration did not materially change the ratio of amino nitrogen to total nitrogen (Table I). Some selective adsorption was indicated by the significant increase in the relative concentration of threonine nitrogen and a decrease in the phenylalanine-nitrogen ratio in one case.

Analysis for threonine was suggested by the known lability of the amino acid in peptide combination (10). The results showed the conditions were sufficiently mild to spare this amino acid even in the enzymatic hydrolysate which contained considerable peptide combinations. The effect of the alkaline conditions probably accounts for the increase in ammonia nitrogen found in both desulfurized preparations.

The reduction of the benzenoid ring of phenylalanine by drastic action of Raney nickel has been reported (11). Chemical analysis on the treated hydrolysates indicated that significant amounts of phenylalanine remained in the desulfurized hydrolysates.

Qualitative evidence for the retention of the principal constituents of the mixtures was found in the distribution patterns of the amino acids on two-dimensional paper chromatograms. These patterns were unchanged as a result of the desulfurization reaction except for the disappearance of the methionine spot and a notable increase in the size of the  $\alpha$ -aminobutyric acid spot. The minimum quantity of each amino acid which will yield a spot with ninhydrin under these conditions has been estimated by Dent (8). The occurrence of a spot for a given amino

acid thus places a minimum limit on the amount retained in the hydrolysate.

Complete chemical or microbiological assay which would quantitatively define the losses of individual amino acids from any treated hydrolysate was not required for our purposes. The presence of each of the essential amino acids, with the exception of methionine, was demonstrated by a simple growth test using young rats. The results of these studies, recorded in Table II, show, as might be expected, that when the desulfurized hydrolysates were used to furnish the source of nitrogen in the diet of the young rats, the animals steadily lost weight. Supplementation of the desulfurized enzymatic hydrolysate with methionine and cystine, but not with cystine alone, produced a prompt resumption of growth. The effect of methionine supplementation of the diet containing the desulfurized acid hydrolysate was not as striking. However, the untreated acid hydrolysate has been found to produce a slow rate of growth in rats at this laboratory. Therefore, the arrestment of the weight loss on the desulfurized diet by the addition of methionine, or methionine and cystine, and a subsequent gain in weight appear to be significant. These growth studies show that none of the other essential amino acids have been removed by the desulfurization process to a level below the minimum requirements for the diet of young rats (12) if the desulfurized hydrolysate is incorporated in the diet at a 20% level. No attempt to obtain optimum growth response, or to make quantitative comparison between treated and untreated hydrolysates has been made.

The desulfurization procedure is probably applicable to any protein hydrolysate. Preliminary experiments indicated that the procedure described in the experimental section is insufficient to completely remove all the sulfur from intact proteins such as casein and egg albumin.

### SUMMARY

A procedure for preparing sulfur-free protein hydrolysates has been presented. A mildly alkaline solution of the hydrolysate was shaken with Raney nickel catalyst until organic sulfur was converted to nickel sulfide. By this treatment the cystine and methionine were quantitatively destroyed, presumably by conversion to L-alanine and L- $\alpha$ -aminobutyric acid, respectively. This view was supported by two-dimensional paper chromatograms.

The procedure has been demonstrated with an acid and an enzymatic hydrolysate. Chemical tests showed that part of the nitrogenous material remained adsorbed on the nickel. The ratio of amino nitrogen to total nitrogen was not materially changed. Growth tests showed the desulfurized hydrolysates, after the addition of methionine, supported the growth of young rats.

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# Studies of the Nutritive Impairment of Proteins Heated with Carbohydrates. II. *In Vitro* Digestion Studies

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## INTRODUCTION

Reports of studies upon the behavior of proteolytic enzymes toward modified proteins are relatively few. A number of chemical derivatives of edestin were made by Kiesel and Roganawa (1). The results demonstrated that certain groupings in the protein are necessary for full trypsin activity. Yenson (2) has shown that the enzymatic splitting of gluten was impaired by prior heating in butter or olive oil. Work by McChesney and Roberts (3) has indicated that reduction of protein in liquid ammonia by sodium results in derivatives that are less susceptible to trypsin. Davis, Rizzo and Smith found heated lactalbumin to be markedly less digested by pepsin and pancreatin than unheated (4). On the other hand, Eldred and Rodney (5) and Hanks and co-workers (6) report that heated casein was nearly as well digested by pepsin, trypsin, or chymotrypsin as raw casein. Tazawa found that D-lysine anhydride dihydrochloride is split by pepsin but is indifferent to trypsin or papain (7). Roche and Mourgue prepared a series of substituted guanido compounds and found them to be resistant to hepatic arginase (8).

Work in this laboratory by McInroy, Murer and Thiessen (9) has shown that autoclaving moist casein with dextrose results in a drastic lowering of the biological value of the casein as revealed by rat growth bioassay. It is shown herein that certain proteolytic enzymes are unable to split the dextrose-treated casein. The present work indicates that wheat gluten autoclaved in the presence of dextrose is resistant to proteolytic enzymes. It is believed that a protein-sugar reaction has taken place and that this explains the loss in the protein's biological value and the resistance exhibited toward digestive enzymes.

Melnick, Oser and Weiss (10) have put forward the hypothesis that the rate of release of amino acids from a protein undergoing digestion is a key factor in nutrition. The present work indicates this rate of release is disturbed by treatment of the protein by dextrose and heat.

### SOURCE OF EXPERIMENTAL MATERIALS

The pepsin, trypsin and chymotrypsin were crystalline preparations from Armour and Company, Chicago, Illinois. Pancreatin, "crude," and the casein (88.7% protein) were purchased from General Biochemicals Company, Chagrin Falls, Ohio; Rhozyme DX, from Rohm and Haas. The gluten (78.7% protein) and papain were obtained from Nutritional Biochemicals, Cleveland, Ohio; Dextrose was Merck's anhydrous.

### METHOD

#### *Preparation of Materials*

An equal weight mixture of finely ground protein (Kjeldahl N  $\times$  6.38 for casein or  $\times$  5.7 for gluten) and dextrose was mixed with 0.5 ml. distilled water/g. of crude protein. This was autoclaved 2 hr. at 15 p. s. i. and gave rise to a material hereafter referred to as the protein-dextrose complex. Generally, 1 kg. of material was prepared at a time. The dark brown, leathery material was thoroughly dried in an air oven and finely ground in a Raymond Mill. The resulting preparations contained 8% N for gluten and 7.1% N for casein. In order to extract unbound dextrose and water-soluble "melanoidins" a glass column was packed with the complex, and distilled water was

TABLE I  
*Summary of Materials Used*

Symbol	Description	Per cent nitrogen (Kjeldahl)
A	Casein, finely ground in Raymond Mill.	13.9
B	1 g. of casein protein (N $\times$ 6.38) autoclaved with 0.5 ml. water.	13.4
C	1 g. casein protein + 1 g. dextrose + 0.5 ml. H <sub>2</sub> O thoroughly mixed and dried at room temperature.	7.1
D	1 g. casein protein + 1 g. dextrose + 0.5 ml. H <sub>2</sub> O autoclaved 2 hr. at 15 p. s. i., ground in Raymond Mill.	7.99
E	D exhaustively ground in Ball Mill and sifted through No. 6XX silk flour sieve.	7.9
G	D was packed in a large glass tube and exhaustively extracted with H <sub>2</sub> O until washings were only slightly colored (yellow).	11.0
H	Gluten, finely ground in Raymond Mill.	13.9
J	1 g. gluten protein (N $\times$ 5.7) + 1 g. dextrose + 0.5 ml. H <sub>2</sub> O autoclaved 2 hr. at 15 p. s. i.	8.0
K	J was treated similarly to D in yielding G.	13.0
L	Similar to J, however, lysine added to the gluten before autoclaving to the level found in casein.	8.4

passed through until the washings showed only the faintest yellow tinge. Dried and ground, the residue contained 13% N for gluten and 11% N for the casein. Table I contains a summary of the preparations employed.

### *General Method of Enzyme Digestion*

Amounts of the treated protein were weighed out so as to provide equivalent amounts of nitrogen for each digest. These were suspended in phosphate buffer at pH 7.6 (*M*/15 Sorensens) in most cases and pH 1.5 for one pepsin run, Expts. 23-26. These were heated 1 hr. in a boiling water-bath under a condenser to aid in solubilizing the protein. Occasionally, the more difficultly soluble proteins were ground in a glass mortar with successive amounts of buffer before heating. Upon cooling, toluene was

TABLE II

*Protocols of Enzymatic Digestions of Casein-Sugar Complexes\**

Substrate <i>g.</i>	Experi- ment	Enzyme	pH	Amino N/100 ml. digest mg. <i>Hours</i>							
				0	15	2	3	4	45	6	24
A 5.76	6	Trypsin	8.3	6	37	—	39	—	—	42	42
C 11.24	8	Trypsin	8.3	3	32	—	33	—	—	35	45
E 10.0	10	Trypsin	8.3	5	11	—	7	—	—	11	8
E 10.0	12	Trypsin	8.3	7	7	—	7	—	—	11	9
A 2.88	23	Pepsin	1.6	1	—	28	—	43	—	51	62
C 5.62	24	Pepsin	1.6	2	—	39	—	55	—	63	83
D 5.0	25	Pepsin	1.6	5	—	23	—	37	—	48	69
A 5.76	35	Papain	7.5	6	11	—	17	—	18	—	28
D 10.0	37	Papain	7.5	4	5	—	5	—	5	—	10
A 5.76 + 100 mg. hydroxymethyl- furfural	16	Trypsin	8.5	13	23	—	25	—	25	—	33
A 5.76 + 10 g. dextrose auto- claved moist 2 hr.	14	Trypsin	8.5	10	17	—	19	—	21	—	28
A 2.88	31	Pancreatin	7.6	—	—	91	—	106	—	104	125
C 5.62	32	Pancreatin	7.6	—	—	71	—	73	—	83	92
D 5.0	33	Pancreatin	7.6	—	—	53	—	57	—	60	70
G 3.62	34	Pancreatin	7.6	—	—	68	—	86	—	87	96

\* Many additional experiments were carried out. Additional data and details may be obtained from the authors.

added and the pH checked and small adjustments made to bring it to the optimum for the respective enzymes. Papain was activated with bisulfite. The enzymes were added and the digests incubated at 37°. In some cases, the digests were "stirred" by passing in moderate currents of air or nitrogen. The volume of a digest changed but slightly during an experiment.

Aliquots were removed at intervals, as indicated in Table II, filtered, and, either analyzed directly for amino nitrogen, or subjected first to a precipitation with 25% trichloroacetic acid followed by a second filtering. The amino-nitrogen analysis of Pope and Stevens (11) was selected as possessing the double advantage of requiring but 5 ml. of digest and allowing many analyses to be run off in a single day. The method was checked with the Van Slyke apparatus and gave very satisfactory checks.

## RESULTS AND DISCUSSION

Table II records the results of the digestions. The symbols assigned to the various substrates are defined in Table I.

It appears that the major fact brought out in the present work is the resistance exhibited by protein-sugar complexes to the action of trypsin. This is clearly shown in Fig. 1 wherein the casein-dextrose complex resists almost completely the action of trypsin. There, only the results of one digestion are given but many additional experiments involving trypsin show the same effect. Melnick and collaborators emphasize that for full nutritive response to a dietary protein to be achieved, the amino acids must be released at rates whereby they can be utilized fully in the metabolic cycle (10, 18). Assuming that this is true would account for the failure of the casein complex to support growth in young rats (9) providing the *in vitro* blocking of tryptic action herein observed is also true *in vivo*. The rats in question excreted feces which were described as being black and "sticky," strongly suggesting an undigestible ration. Similar cessation of growth is observed following feeding of the well-known trypsin inhibitor of unheated soybeans.

Other agents may play a part. Some loss of activity is apparent when hydroxymethylfurfural, a known heat degradation product from hexoses (12), (Expt. 16) is added to a trypsin digest. Other data have been obtained that indicate the compound inhibits trypsin *in vitro*, and tentative results suggest its presence in the casein-dextrose complex (13). The substance arrests the growth of rats when fed at levels of 2 mg./day (14).

No such trypsin "inhibition" was forthcoming with casein-dextrose mixed and dried at room temperature (Expt. 8). Casein autoclaved

with water alone is well digested by trypsin which is a confirmation of other work (5, 6). Davis, Rizzo and Smith (4) found heated lactalbumin to be a poor growth supporting protein, and pepsin plus pancreatin gave rise to only limited release of soluble nitrogen. The effect of heat upon lactalbumin is possibly due to the presence of naturally occurring lactose in the protein. Such is not the case with casein.

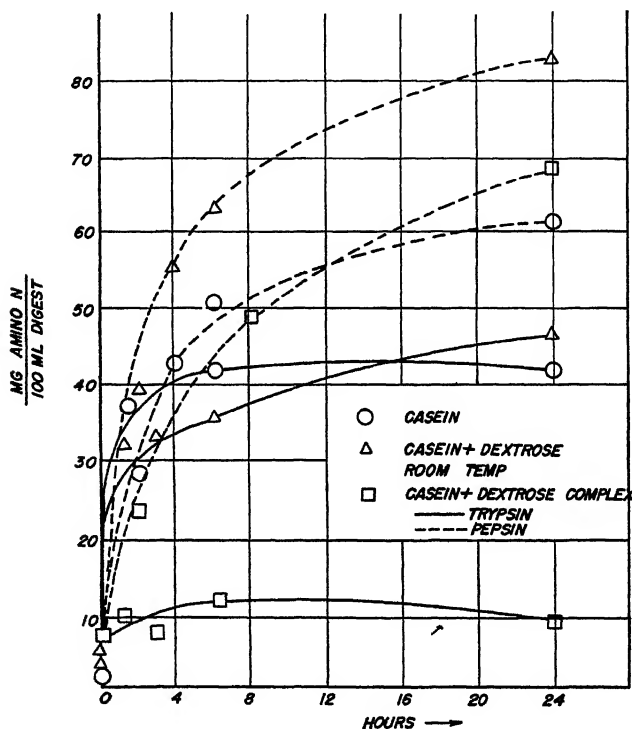


FIG. 1. Comparison of rates of digestion of casein, casein-dextrose dried at room temperature, and casein-dextrose complex by pepsin and trypsin. See Table II, Expts. 6, 8, 10, 12, and 23, 24, 25.

Pepsin does digest the casein complex as can be seen in Fig. 1 and Table II (Expts. 23-25). The gluten-dextrose complex is also attacked by pepsin (Fig. 3). Chymotrypsin appears to increase the rate of proteolysis of the complex but further work is needed with this enzyme. Lenti presents evidence which points to both pepsin and chymotrypsin being able to attack the same linkages in a protein (16).

Pancreatin is about half as effective against the complex as against untreated casein; see Table II (Expts. 31 and 33). Since here a mixture of enzymes is at work, little can be said as to the specific linkages split. One can visualize a digestion during which a large increase of amino nitrogen is observed with none of sugar blocked linkages being split.

The interaction of amino acids and sugars has been extensively investigated, still no clear picture has yet emerged as to the precise

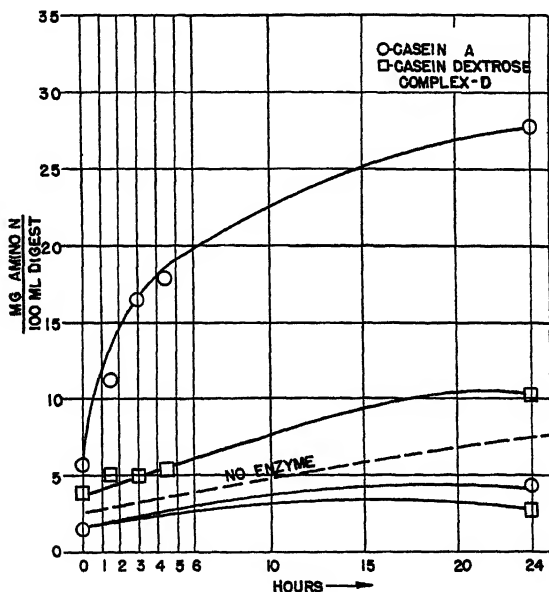
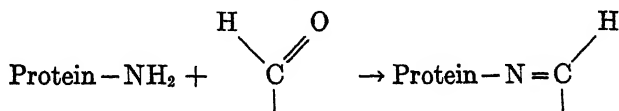


FIG. 2. Rate of papain digestion of casein and casein-dextrose complex.  
See Table II, Expts. 35 and 37.

chemistry of the reaction. Von Przylecki and Chichocka list no less than eight possible ways by which combination can occur (15). The simplest representation of the classic Maillard reaction is:



The amino groups pictured here would arise from the terminal (free) amino groups of an amino acid such as lysine. Other groupings

are undoubtedly able to react with dextrose. For example, carboxyl groups from the protein could enter into ester linkages while hydroxyl-containing amino acids could give rise to ethers. Imino compounds are possible reactive centers.

As a tentative picture of what has taken place to block the trypsin we employ the conclusions arrived at by Bergmann and co-workers (17).

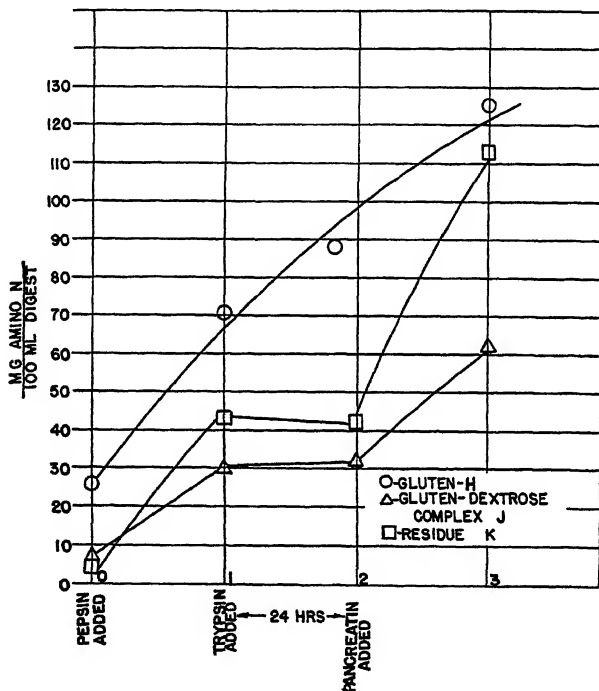


Fig. 3. Digestion of gluten, gluten-dextrose complex, and residue by pepsin, trypsin, and pancreatin.

The structure of the enzyme substrate is vital and amino groups in the side chain are important for the activity of trypsin. It is assumed that when such groups are in combination with dextrose residues the enzyme is blocked off from the substrate, resulting in a definite loss of proteolysis. Work involving benzoylated and deaminated edestin has indicated rather conclusively the vital role played by amino groups in the action of trypsin (1) (2). Such side chains resemble lysine and

arginine. Lysine in the protein is especially important since a portion of its amino groups are "free" and able to combine with reducing sugars. Autoclaving a protein low in lysine, such as zein, with dextrose yields a light-colored product that is digested relatively well by trypsin (13).

Such is apparently the case also for papain and the present work indicates this enzyme is also blocked by the entering dextrose (Fig. 2). Papain is an enzyme that attacks linkages similar to those split by trypsin, and here also the requisite side chains contain free amino groups. Reaction of such amino groups with dextrose hinders the proteolytic action of papain.

The side-chain groups necessary for pepsin action are considerably different from the corresponding ones for trypsin action. The possibility presents itself that in the chemical nature of these side-chain groups resides the explanation for the difference observed in proteolytic activity of pepsin and trypsin. It is significant that the side-chain groups thought necessary for pepsin activity are characterized by the presence of the far less reactive benzene or phenolic rings. Dextrose reacts far more slowly or not at all with such groups, and blocking of the enzyme does not take place.

Prior digestion of either casein or gluten complex with pepsin does not render the modified protein subject to attack by trypsin. Figure 3 indicates that when the enzymes are employed in physiological sequence and at the appropriate pH, trypsin is still unable to attack the protein-dextrose complex. Similar data are available for casein-dextrose complexes.

#### ACKNOWLEDGMENTS

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#### SUMMARY

The autoclaving of moist casein or wheat gluten with dextrose has been shown to give rise to a protein-sugar complex that is digested *in vitro* by pepsin, chymotrypsin, and pancreatin but is resistant to the action of trypsin and papain. This is discussed in the light of Bergmann's hypothesis that enzymes require specific groups in the substrate for activity. It is suggested that the reaction of the sugar with the protein blocks the necessary groups, and tryptic activity is no longer

possible in the modified protein. The nutritional importance of the loss of digestibility is pointed out.

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# The Inactivation of Insulin by Tissue Extracts. IV. Changes in Insulin Sensitivity in Rabbits Induced by Previous Injections of Rat Liver Extracts <sup>1</sup>

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## INTRODUCTION

During the course of experiments (1) designed to assay the activity of the insulin-inactivating system (insulinase) present in mammalian livers (2) by means of the rabbit insulin assay method, the details of which have been described in other reports (1, 3, 4), a great degree of variability was noted in the insulinase activity of livers obtained from adult male rats. Further investigation of this phenomenon indicated that the major part of the variability was attributable to progressive changes in the insulin sensitivity of the test rabbits used for assay purposes. This report is concerned with the effect of repeated intravenous injections of rat liver extracts on the insulin response of the rabbit.

## METHODS

An experiment which was originally designed to test the effect of three types of diets on the insulinase activity of rat liver may be used to illustrate the alterations of insulin sensitivity induced in rabbits by weekly injections of rat liver extracts. Since the method employed for this purpose will be described in detail elsewhere (1), only the basic principles of the assay procedure need be noted. Liver extracts were prepared and their insulinase activities assayed according to procedures outlined elsewhere (3, 4), with the following modifications: One ml. of extract was incubated for 30 min. at 37°C. with 100 units of amorphous insulin <sup>3</sup> contained in 1 ml. of

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<sup>2</sup> With the technical assistance of Gladys Perisutti.

<sup>3</sup> We are indebted to the Eli Lilly and Co. for generous supplies of a highly purified lot of amorphous insulin containing 22.5 units/mg.

solution. At the end of this period, two aliquots of the reaction mixture were diluted with water; one aliquot was diluted 1:35, the other 1:60. Dilution to this extent has been demonstrated to stop the destruction of insulin as well as to dilute the residual quantity of insulin to levels appropriate for the rabbit assay.

For the estimation of the amount of insulin that remained in the reaction mixture at the end of incubation, each of five fasted, previously unused rabbits was injected intravenously with 1 ml. of a 1:35 dilution of the incubation mixture to be tested, and each of five other rabbits was injected with 1 ml. of the 1:60 dilution. Each milliliter of these two dilutions contained the extractable materials of 4.7 mg. and 2.75 mg., respectively, of fresh liver. Blood samples were obtained before injection (fasting blood sugar) and at 50 min. after injection (5), and their glucose content was determined by the Nelson modification of the Somogyi procedure (6).

The rabbits were injected according to a Latin square design which permitted the use of a crossover type of schedule, the essential details of which are summarized in Table I. Animals which had once been injected with either one of the two dilutions were not subsequently crossed with animals that had been injected with the other dilution. There were thus established two distinct series of crosses—one involving the 1:35 dilutions, the other involving the 1:60 dilutions. The three phases of each cross were performed on the same days of three successive weeks.

TABLE I

*Design of Assay Indicating the Source of Rat Liver Extracts Injected in the Various Reaction Mixtures*

Day of week	Dilution of reaction mixture							
	1:35				1:60			
	Rabbit group	Week of experiment			Rabbit group	Week of experiment		
		First week	Second week	Third week		First week	Second week	Third week
Monday (first day)	A	I	II	III	A'	I	III	II
	B	II	III	I	B'	II	I	III
	C	III	I	II	C'	III	II	I
Tuesday (second day)	D	I	III	II	D'	I	II	III
	E	II	I	III	E'	II	III	I
	F	III	II	I	F'	III	I	II

Roman numerals refer to the group of rats receiving one of the 3 dietary treatments investigated. The reaction mixtures, containing liver extracts prepared from a single rat of the designated group, were injected into each of the 5 individuals comprising the various groups of rabbits. The sequence of injections on Wednesdays and Fridays was similar to that used on Monday. The sequence on Thursdays was similar to that used on Tuesday.

In this type of design, the mean hypoglycemic response of all of the rabbits injected during the first week should be the same as the mean responses obtained from all of the animals injected during the second week and during the third week of the cross. This will be true regardless of any differences in the amounts of insulin contained in the three solutions being assayed and irrespective of the degree of variability in the insulin sensitivities among the different rabbits. However, the mean hypoglycemic response during each phase of the cross will be affected by any progressive changes in the insulin sensitivities in individual rabbits.

## RESULTS

For reasons to be considered below, not all of the rabbits injected during the first week survived the entire 3-week crossover schedule. Complete results of all three crosses were obtained from 35 rabbits

TABLE II  
*Effect of Successive Injections of Reaction Mixtures on the Apparent  
Insulinase Activity of Rat Liver Extracts*

Week of experiment	Dilution of reaction mixture injected			
	1:35		1:60	
	Number of rabbits injected	Insulinase activity (% of fasting blood sugar)	Number of rabbits injected	Insulinase activity (% of fasting blood sugar)
First (rabbits previously not injected)	35	65 $\pm$ 15.16	39	72 $\pm$ 16.81
Second (rabbits previously injected once)	35	60 $\pm$ 13.89	39	64 $\pm$ 18.06
Third (rabbits previously injected twice)	35	52 $\pm$ 20.19	39	61 $\pm$ 20.12
Significance of differences	Highly significant ( $p < 0.01$ )		Significant ( $0.05 > p > 0.01$ )	

Insulinase activities expressed in terms of mean  $\pm$  standard deviation.

which received injections of the 1:35 dilutions of the reaction mixtures; results from 39 rabbits were available for determination of the effect of the three weekly crosses of the 1:60 dilutions. In the case of these 74 rabbits, the results obtained from analysis of the 50-min. samples

were calculated in terms of the blood sugar in actual mg.-%, the mg.-% drop from the fasting level, and as a percentage of the fasting level. In deference to brevity, the results will be expressed usually only in terms of percentage of the fasting level. However, expression of the results by either of the other two methods did not alter the significance of the observations in any respect whatsoever.

The data in Table II clearly demonstrate the apparent progressive decrease in insulinase activity during each succeeding week. The

TABLE III  
*Effect of Successive Injections of Reaction Mixtures  
on Fasting Blood Sugar Levels*

Week of experiment	Dilution of reaction mixture injected			
	1:35		1:60	
	Number of rabbits injected	Fasting blood sugar (mg.-%)	Number of rabbits injected	Fasting blood sugar (mg.-%)
First (rabbits previously not injected)	35	100 $\pm$ 14.71 <sup>a</sup>	39	100 $\pm$ 14.99 <sup>a</sup>
Second (rabbits previously injected once)	35	83 $\pm$ 11.58	39	83 $\pm$ 11.09
Third (rabbits previously injected twice)	35	73 $\pm$ 16.92	39	74 $\pm$ 15.81
Significance of differences	Highly significant (p < 0.001)		Highly significant (p < 0.001)	

<sup>a</sup> Fasting blood sugars expressed in terms of mean  $\pm$  standard deviation.

differences in the mean values obtained during each of the 3 weeks were found to be highly significant when subjected to an analysis of variance (7). Just as striking was the progressive fall in the mean fasting blood sugar values during these three weeks (Table III). The differences between these mean fasting blood sugar values were also found to be highly significant.

The lower fasting blood sugars of the previously injected rabbits were particularly striking. A fasting blood sugar of less than 60 mg.-% was not encountered in a single instance in any of the 150 previously

uninjected rabbits employed during the first week of the experiment. Five rabbits which had received one previous injection were observed to possess fasting blood sugars below this level during the second week of the experiment. During the third week of the cross, 15 rabbits, all of whom had been injected during each of the two previous weeks, were found with fasting blood sugars lower than 60 mg.-%.

Thirty-nine of the rabbits failed to survive the 50-min. postinjection periods of observation of all three phases of the cross. Of this total of 39, nine expired during the course of the actual assays, so that an opportunity was presented to estimate their fasting blood sugars almost immediately before death. Seven of these rabbits had been used in the crosses for injection of the 1:35 dilutions and two had been injected with 1:60 dilutions. Of these 9 rabbits that died during the actual assays, five had fasting blood sugars lower than 40 mg.-%. All five of these rabbits succumbed before the reaction mixtures could be injected. The other 4 rabbits with higher fasting blood sugars died subsequent to the injections. Two of these are known to have died during typical hypoglycemic convulsions induced as a result of the injections.

Two types of experiments were performed in an effort to reveal the cause of the apparent progressive decrease in the insulinase activities of the extracts during each of the 3 weeks of the injection schedule. On the one hand, a number of preliminary experiments indicated that when reaction mixtures, prepared from the livers of similarly treated rats, were injected into a series of previously *unused* rabbits, no progressive changes in insulinase activity were observed over a period of time. On the other hand, the fact that successive injections of the liver extracts produced profound alterations in the insulin sensitivity of these rabbits was easily demonstrated by comparison of insulin tolerance curves in these animals with those obtained from rabbits which had received no previous injections of liver extracts.

The insulin tolerance tests were performed after a preliminary overnight fast of 18 hr. Each rabbit was given an intravenous injection of crystalline insulin <sup>4</sup> at a dosage level of 0.2 units/kg. of body weight, and blood samples were obtained, before injection (fasting blood sugar), and at 30, 60, 90, and 120 min. thereafter. Such tests were performed on 25 of the rabbits ("used rabbits") which had previously received

<sup>4</sup> We are indebted to the Eli Lilly and Co. for generous supplies of this lot of crystalline insulin containing 40 units/ml.

3 injections of rat liver extract at weekly intervals during the course of the feeding experiment outlined above. Fifteen healthy, adult, previously "unused rabbits" were also subjected to the same test.

The results of these insulin tolerance tests are presented in Table IV and Fig. 1 and indicate that the "used rabbits" were definitely more sensitive to insulin than the "unused rabbits." Comparison of the results in Table IV for the two groups of rabbits at each of the time intervals following insulin administration revealed that the depression of the blood sugar, expressed in terms of percentage of the fasting level, induced by the administration of the insulin was significantly

TABLE IV  
*The Effect of Previous Injections of Reaction Mixtures on Insulin Tolerance Tests in Rabbits*

History of rabbits	Body weights	Fasting blood sugar	Blood sugar levels after injection of insulin (% fasting level)			
			30 min.	60 min.	90 min.	120 min.
Normal rabbits (previously uninjected)	kg.	mg.-%				
	1.4 $\pm$ 0.102	87 $\pm$ 10.22	105 $\pm$ 14.24	83 $\pm$ 19.42	80 $\pm$ 19.50	100 $\pm$ 20.23
"Used" rabbits (previously injected on each of 3 successive weeks)	1.6 $\pm$ 0.446	67 $\pm$ 10.97	55 $\pm$ 22.64	55 $\pm$ 21.73	68 $\pm$ 21.72	83 $\pm$ 24.30
Significance of difference	Not significant (0.2 > p > 0.1)	Highly significant (p < 0.001)	Not significant (0.2 > p > 0.1)	Highly significant (p < 0.001)	Highly significant (p < 0.01)	Significant (0.05 > p > 0.01)

All values stated in terms of mean  $\pm$  standard deviation.

greater at the 60, 90, and 120-min. intervals in the group of "used rabbits" which had received previous injections of liver extract. The values for the 2 groups at the 30-min. interval were not significantly different. Inspection of Fig. 1, in which the results are expressed as actual mg.-% drop below the fasting levels, reveals that the blood sugar curve of the "used rabbits" did not return to the level of the fasting value by 120 min. Further examination of the curves demonstrated that the slopes of the two curves were roughly parallel between the 60 and 120-min. intervals, although the actual blood sugar values differed significantly at each point. These facts indicate that the rates of fall of the blood sugar were approximately equal in the two groups

of animals but that the "insulin effect" was more prolonged in the case of the used rabbits. However, there did not appear to be any difference between the two groups with regard to the mechanisms responsible for the restitution of the depressed blood sugar.

In order to seek some explanation for the above findings, various preliminary studies were performed on the same rabbits used for the preceding two experiments. Liver glycogen was determined in some rabbits but no consistent differences were noted in the two groups of

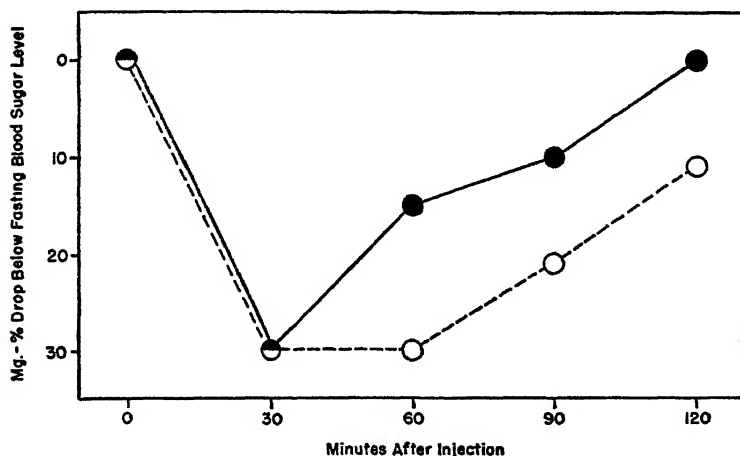


Fig. 1. Effect of previous injections of rat liver extracts on insulin tolerance curves in rabbits. The two lines represent the mean blood sugar responses of each group to the test dose of insulin. The black circles represent the "unused" rabbits; the white circles represent the previously-injected rabbits.

animals. Intravenous glucose tolerance tests gave similar results in the two groups of rabbits. Gross and microscopic examination of the liver, pancreas, adrenals, and other tissues of the "used rabbits" showed no significant pathologic changes.<sup>5</sup> Serum obtained from the "used rabbits" was tested for an insulinase inhibitor or antibody by incubation of the serum with insulin and active rat liver extract, but was found not to inhibit the *in vitro* inactivation of insulin by the rat liver extracts. Finally, assay of the insulinase activity of the livers

<sup>5</sup> The tissues and sections were examined by Dr. Philip Wasserman, Director of Clinical Laboratories, The Jewish Hospital.

obtained from "used" and "unused" rabbits revealed no consistent differences in this respect between the two groups of animals.

### DISCUSSION

The observations noted herein reveal that there is a progressive fall in the fasting blood sugar of the rabbit following repeated intravenous injections of rat liver extracts. This decrease is not dependent upon the presence of the insulin contained in the reaction mixtures injected since the same phenomenon could be produced by the injection of extracts without added insulin.

A progressive increase in the sensitivity of the rabbits to exogenous insulin has also been observed. This has been demonstrated both by the altered hypoglycemic responses of the same rabbits to the successive injections of reaction mixtures containing approximately equivalent quantities of insulin, and by the increased sensitivity of the injected rabbits in insulin tolerance tests, as compared with previously uninjected animals. As the insulin tolerance tests reveal, the same dosage of insulin maintains the blood sugar at a low level for a longer period of time in the previously injected rabbits.

The mechanism responsible for the increased insulin sensitivity is obscure. It appears probable that it is a consequence of the state of "hypersensitivity" induced as the result of the injection of protein extracts. Certainly, rat liver extracts are not unique in their ability to invoke this phenomenon. We have observed the same increase in insulin sensitivity in rabbits repeatedly injected with human plasmin prepared by the activation of Fraction III of human plasma protein by streptokinase (8). Quite similar phenomena have been reported by others to have occurred in response to the injection of bacterial products such as *H. pertussis* vaccine into mice (9) and meningococcus and *Salmonella* endotoxins into rabbits (10). On the other hand, some of our preliminary observations indicate that injections of the fraction of rat liver, soluble in 70% ethyl alcohol or in acetone, fail to alter the insulin sensitivity of the rabbit. The reaction therefore appears to be part of a nonspecific response to the injection of a foreign protein although this has not been established with certainty.

It is questionable if the response is part of the state of anaphylactic shock. A few of the rabbits in these experiments may have exhibited the rather atypical symptoms of this state. As was noted above, only

nine of the rabbits died or showed any symptoms other than those due to hypoglycemia during the period immediately following the injections. Most of the fatal cases died as long as 7 days after the preceding injection. There is also reason to suspect that a single injection of the rat liver extract can alter the rabbit's response to a subsequent injection of insulin alone.

Although the changes produced by the injections must be profound, the rabbits exhibited relatively few signs of these prior to death. The most obvious symptoms were listlessness and a gradual loss of weight. A total of 90 rabbits that had died following previous injections of rat liver extracts have been subjected to routine gross and histopathologic study. In the overwhelming majority of these rabbits, no lesions were found other than those occasionally seen in stock laboratory rabbits.

It is believed that most of these rabbits died as the result of a severe hypoglycemia. This hypothesis is in accord with the extremely low fasting blood sugars encountered in the occasional rabbit which expired during the course of an assay proper and prior to injection of the reaction mixture. It seems probable that, as a result of the injections, the animals developed anorexia as manifested by their gradual weight loss. In the presence of the combination of a marked tendency towards a low fasting blood sugar and the failure to ingest enough food, the blood sugar would tend to drop rapidly and the animals might die in profound hypoglycemia without visible lesions.

Certain of the data obtained from a study of these rabbits render improbable some of the more obvious explanations that could be advanced to account for the effect of the injections of the extracts. The absence of gross and microscopic lesions in the livers of the rabbits exhibiting insulin hypersensitivity, as well as the normal intravenous glucose tolerance test and liver glycogen levels in these animals, fails to provide any evidence for the existence of a significant degree of functional liver impairment. The grossly and microscopically normal adrenal glands of these rabbits argue against the development of insulin sensitivity as the result of an adrenal cortical insufficiency which could conceivably have been induced by repeated injections of noxious foreign proteins.

A shift in the balance between insulin production and insulin destruction may well result in an alteration of insulin sensitivity. An in-

creased production of insulin in the presence of a normal rate of insulin destruction, or a diminished rate of insulin destruction along with a normal rate of insulin production by the pancreas, represent ways in which a state of increased sensitivity to insulin may be produced. However, no gross or microscopic lesions were noted in the pancreas of any of the injected rabbits. This fact by itself does not, however, necessarily indicate a normal rate of insulin secretion by the pancreas. The insulin-inactivating capacity (insulinase activity) of the livers of the insulin sensitive rabbits was found not to differ consistently from that of normal rabbits. If the insulinase activity of the liver is an accurate index of the rate of destruction of insulin *in vivo*, this last fact implies the absence of any change in the rate of insulin destruction in the insulin sensitive rabbits.

Regardless of the nature of the intimate mechanism involved, the striking progressive decline in the fasting blood sugar of the "used" rabbits can undoubtedly be attributed to the same processes that result in an increase in their sensitivity to exogenous insulin. In other words, the injections of rat liver extracts are believed so to modify the response of the rabbit to insulin that the animal becomes more sensitive to his own, endogenous supply of this hormone. According to this explanation, the pancreas of the "used" rabbit is assumed to be producing insulin at its customary level.

Since related observations have been reported in mice (9), it is natural to speculate whether a similar state can be produced in man. To the best of our knowledge, however, there are no notations of this type of observation in man in spite of the tremendous number of individuals who must have received injections of vaccines, sera, and other foreign proteins and who have subsequently been studied for aberrations of carbohydrate metabolism.

The most important practical implications of these observations concern their bearing on the assay of insulin in the presence of contaminants capable of invoking the phenomenon described in this report. Under such circumstances, conventional designs for the assay of insulin obviously may lead to altogether confusing results. Since most assays are performed with either "pure" insulin or with material extracted with 70% acidified alcohol, the injections do not contain whatever substance is responsible for the profound alteration in insulin sensitivity.

## SUMMARY AND CONCLUSIONS

The repeated intravenous injection of small doses of an incubated mixture of rat liver extract and insulin into healthy adult male rabbits resulted in a progressive increase in their sensitivity to insulin and a progressive decrease in their fasting blood sugar. The same phenomenon could be induced by the injection of liver extracts without added insulin.

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# The Chloride Content of Frog Muscle

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## INTRODUCTION

There is apparently some uncertainty as to the amount of chloride which is contained in frog muscle. For although some authors, Van Slyke (1), Wilson and Ball (2), Cullen, Wilkens and Harrison (3), Cameron and Walton (4), and Sunderman and Williams (5), obtained results which are in fair agreement. Heilbrunn and Hamilton (6) have questioned the validity of the analyses.

In determining chloride, the common practice is to subject the muscle to wet washing. However, there has been growing evidence that this method of analysis is inadequate for muscle. In his original paper, Van Slyke (1923) stated that "the accuracy of the method with tissues has not been demonstrated as completely as with blood." Heilbrunn and Hamilton (6) analyzed water extract of muscle and obtained higher chloride values than could be obtained by wet washing. They also tested the Van Slyke technique by adding chloride to muscle which had been rendered chloride-free as tested by the Van Slyke method, and they found an average loss of 25.5% of chloride in the titration. Also, in an earlier paper, C. B. Davis, M. E. Hanke and G. M. Curtis (7) reported that "in the analysis of rabbit tissue the Van Slyke method does not recover all of the chloride present."

It would seem therefore that new values for chloride need to be established. In the Parr bomb method, there is no possibility of chloride loss. Accordingly this method was used for determining the chloride content of muscle and the results obtained were compared with the results obtained with the Van Slyke method.

## METHOD

Both wet and dry muscle were used in the analyses. In analyzing wet muscle, the frog was pithed, muscle removed, divided, and weighed. One portion was immedi-

TABLE I

*The Chloride Content of Small Pieces of Wet Frog Muscle*Determined by the Van Slyke method and the Parr bomb method.  
All muscles wet weight.

Experiment no	Wt. of muscle	Millieq./kg. Van Slyke method	Wt. of muscle	Millieq./kg. Parr method
	<i>mg.</i>		<i>mg.</i>	
1	206	18.4	250	35.7
2	203	14.4	195	33.4
3	208	22.9	352	38.8
4	305	12.9	239	39.7
5	202	18.9	245	43.0
6	208	19.4	235	37.3
7	200	18.0	210	40.1
8	199	20.1	301	42.5
9	450	18.0	242	41.8
10	360	24.1	205	53.4

Average Van Slyke method 21.0.

Average Parr method 39.4.

ately burned in the Parr bomb, the other digested according to the standard Van Slyke procedure.

The principle of the Parr bomb method, as described by Parr (8), consists of burning the muscle in the presence of sodium peroxide. In order to accomplish the burning of wet muscle in the Parr bomb, the charge, consisting of sodium peroxide, potassium

TABLE II

*The Chloride Content of Ground Dry Frog Muscle*

Determined by Van Slyke and the Parr bomb method.

Experiment no.	Wt. of muscle	Millieq./kg. Van Slyke method	Wt. of muscle	Millieq./kg. Parr method
	<i>mg.</i>		<i>mg.</i>	
1	200	45.1	205	139.6
2	200	39.4	205	132.1
3	310	66.8	223	139.1
4	410	59.3	200	135.0
5	329	76.2	203	126.1
6	200	57.4	206	121.9
7	200	57.8	206	124.8
8	200	55.9	206	123.7
9	200	57.2	206	126.7
10	200	56.7	206	125.5

Average Van Slyke method 57.2.

Average Parr method 130.3.

nitrate, and benzoic acid, is first thoroughly mixed on a watch crystal. The ignition cup is then layered with this mixture; the muscle is carefully centered in the cup and the remaining charge added and ignited. After ignition, the solid mass of residue is dissolved in warm distilled water; neutralized with concentrated nitric acid; 5 ml. of excess acid is then added. After the addition of the controlled amount of silver nitrate, the solution is boiled for 1 hr. to coagulate the silver chloride. It is cooled to room temperature, filtered, washed twice, and the washings combined with the filtrate. The filtrate is then titrated with 0.01 *N* ammonium thiocyanate until one drop causes a color change which persists for 1 min. at room temperature.

Dry muscle was prepared for analysis by grinding in a meat chopper, and vacuum drying over  $P_2O_5$  to constant weight.

The Volhard titration was used throughout. Five blanks were run on the chemicals used in the Parr bomb and the charge was found to contain 3.2 millieq. of chloride/kg. This value has been subtracted from values listed.

## RESULTS

The results from the analyses of wet muscle, as described, are shown in Table I, and the results for dry muscle in Table II.

## SUMMARY

1. Two methods for detecting chloride in muscle have been compared.

2. In frog muscle, the Van Slyke technique gave values of 21.0 millieq. of chloride/kg. of wet muscle, whereas the Parr bomb determination gave an average of 39.4 millieq./kg. for a portion of the same muscle. Dry muscle averaged 57.2 millieq./kg. of chloride using the Van Slyke procedure and 130.35 millieq./kg. using the Parr bomb method.

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# Kinetics of the Enzyme-Catalyzed Oxidation of Lactic Acid <sup>1</sup>

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## INTRODUCTION

It is now established<sup>1</sup> (1) that the autoxidation of lactic acid in the presence of the lactic dehydrogenase enzyme system, and of a carrier dye such as methylene blue, proceeds by the following steps:

- 1) Lactic acid + coenzyme I  $\rightarrow$  pyruvic acid + reduced coenzyme I.
- 2) Reduced coenzyme I + prosthetic group of flavoprotein  $\rightarrow$  coenzyme I + reduced prosthetic group of flavoprotein.
- 3) Reduced prosthetic group of flavoprotein + dye  $\rightarrow$  prosthetic group of flavoprotein + reduced dye.
- 4) Reduced dye +  $\frac{1}{2}\text{O}_2 \rightarrow$  dye +  $\text{H}_2\text{O}$ .

Reaction 1 requires the apoenzyme of lactic dehydrogenase, and Reaction 2, and perhaps Reaction 3, the apoenzyme of diaphorase flavoprotein. Many qualitative and a few quantitative investigations have been carried out on the kinetics of the system. In the present work, a detailed study has been made of Reaction 1, and an enzyme model has been proposed to explain the main features of the results.

The equilibrium in the system lactic acid + coenzyme I  $\rightleftharpoons$  pyruvic acid + reduced coenzyme I lies very much to the left (2), so that in order for the kinetics to be studied the pyruvic acid produced must be removed. In the literature there are many references to the inhibition of the reaction by pyruvic acid, although it does not seem to have been established that there is any effect other than the one of mass-action;

<sup>1</sup> Abstracted from a dissertation submitted by Sister Irene Marie Socquet, S. S. A. (present address: Anna Maria College, Marlboro, Mass.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry at The Catholic University of America.

however other similar ions such as salicylate and citrate have been shown to be true inhibitors (3). The effect of pyruvate can be obviated by the addition of an excess of cyanide ions, which react to form the cyanhydrin; in addition hydroxylamine and hydrazine have been used. Cyanide has been shown not to inhibit the action of the enzyme, and has been used in the present investigation; the *in vivo* poisoning of the reaction by cyanide is probably due to its effect on the cytochrome-cytochrome oxidase system (4).

The quantitative data in the present investigation were obtained using the ordinary Warburg manometric method. This has the disadvantage of giving a measure of the rate of the overall process, and not that of reaction (1), but by a suitable choice of concentrations of the various components of the system, and by taking certain precautions to be indicated later, it was possible to obtain results which refer to Reaction 1. The same technique was used by Green and Brosteaux (2) to obtain valuable qualitative information on the influence of lactate concentration, coenzyme concentration, pH, and of other factors on the rate of reaction; our results indicate, however, that under some conditions these workers were in reality measuring the rate of reduction of the dye, *i. e.*, of Reaction 3. Straub (5) used the same technique in another study of the influence of various factors on the rate, and obtained the interesting result that with increasing concentration of lactic dehydrogenase the reaction rate rises to a maximum value and then decreases.

We have confirmed this result under somewhat different conditions, and have established the fact that the rate being measured is that of Reaction 1. In the present investigation it has also been shown that the rate of Reaction 1 passes through a maximum with increasing coenzyme concentration, but rises to a limiting value with increasing lactic acid concentration. We explain these results quantitatively in terms of a model (6) according to which, for reaction to occur, the lactic acid and coenzyme must become adsorbed side by side on the apoenzyme; it is moreover postulated that there is competitive adsorption of the coenzyme on the lactic acid sites, but that there is little adsorption of lactic acid on the coenzyme sites. It is further shown that the variations of the activation energy with the various concentrations provide evidence as to the thermodynamic properties of these apoenzyme-substrate-coenzyme complexes.

## EXPERIMENTAL

### *Materials*

The two enzymes lactic dehydrogenase and diaphorase flavoprotein were extracted together from calves' hearts, according to the method of Green and Brosteaux (2); it was concluded on the basis of numerous preparations that the best results were

obtained with hearts weighing over 1.5 lb. and treated within 2 hr. of slaughter. The enzyme extract, if kept in a refrigerator, could be used up to 8–10 days, after which it was too inactive.

The coenzyme was purchased from Schwarz Laboratories, and the solution was freshly prepared each day. The potassium cyanide solution was also prepared daily, and was neutralized with hydrochloric acid to pH 7.2; it was kept in the refrigerator to inhibit hydrolysis. The lactate solution was prepared by diluting lactic acid about 3 times with water and boiling for 10 min. to depolymerize it; sodium hydroxide, dissolved in the minimum amount of water, was then added in stoichiometric proportions. The solution was then titrated with hydrochloric acid to pH 7.3. This solution was also kept cool, to prevent the growth of molds.

The phosphate buffers were prepared according to the specifications of Clark (7). The methylene blue was purchased from the La Motte Chemical Products Company.

In the preparation of all of the solutions, water redistilled in an all-glass apparatus was used; there is, however, no positive evidence that the enzymes concerned are inhibited by heavy metals.

### *Kinetic Procedure*

Three main methods were employed to follow the rate of the reaction under various conditions, and of these the manometric technique was the only one that was successful from a quantitative standpoint. An attempt was made to employ an electrochemical procedure and follow Reaction 1 by measuring the rate of change of the ratio of reduced coenzyme to coenzyme, a potential mediator being introduced into the system; however it was found to be impossible to adjust the conditions so that the potential mediator would come to equilibrium rapidly, and at the same time avoid the re-formation of coenzyme I by Reaction 2. The occurrence of Reaction 2 also introduced difficulty into an attempt to follow spectrophotometrically the formation of reduced coenzyme, and it was not found possible to avoid this by deactivating the flavoprotein without deactivating the dehydrogenase. Moreover, owing to the interference of substances such as the enzymes and the cyanide, the spectrophotometric study could not be made directly on the reaction mixture, and these substances could not be removed quantitatively. That the most important factor causing the failure of these methods is the fact that the coenzyme is re-formed by Reaction 2 was suggested by the result that the iodine titration method of Drabkin and Meyerhof (8) revealed no detectable amount of reduced coenzyme as the reaction proceeded.

The manometric work was carried out in a Warburg apparatus supplied by the American Instrument Company, and the procedure recommended by Umbreit, Burris and Stauffer (9) was followed. The shaking mechanism was regulated to the maximum rate of about 118 strokes/min., the amplitude being 4 in. The total volume of the reaction mixtures was 2.8 ml., and 0.3 ml. of 40% KOH was placed in the inset. Ten minutes were allowed to establish equilibrium with the bath, which was maintained at a temperature accurate to  $\pm 0.05^\circ\text{C}$ .

### RESULTS

Figure 1 presents representative data obtained by the manometric method. The initial lactate concentration is  $a$ , in moles per liter, and  $x$

is the lactate concentration after time  $t$ ;  $\log (a - x)$  is plotted against the time, and the points are seen to be accurately linear during the early stages of the reaction. This indicates that the reaction is of the first order, obeying the law,  $\ln [a/(a - x)] = kt$ . The specific rate constants and the initial rates were calculated from the slope of the line drawn through the experimental points.

A linear relationship between  $\log (a - x)$  and  $t$  was found in all of the runs except when the conditions were such that the rate being

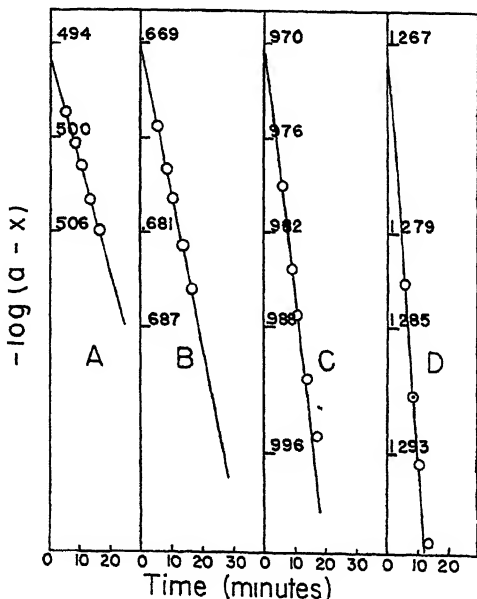


FIG. 1. Plots of  $\log (a - x)$  against  $t$  for various initial lactate concentrations, viz. (A) 0.32 M, (B) 0.214 M, (C) 0.107 M, (D) 0.054 M.

measured was evidently not that of Reaction 1. In particular it was found that unless proper precautions are taken the third and fourth step may become rate-determining. The first possibility, the slow reduction of the dye by Reaction 3, may be eliminated by using a sufficiently high concentration of the dye. Our dye concentrations were all higher, except in exploratory investigations, than those used by previous workers, and we have confirmed that under these conditions the rate is independent of the dye concentration and of the type of dye

used; Reaction 3 cannot therefore be rate-controlling. That the last step may sometimes become rate-controlling is demonstrated by decoloration of the dye during the course of reaction. This difficulty was avoided by using the maximum rate of shaking. Only data obtained in investigations in which these precautions were taken are quoted in this paper.

In Fig. 2 are shown initial rates,  $v$ , plotted against concentration of lactate, at a series of temperatures, and corresponding to an optimal

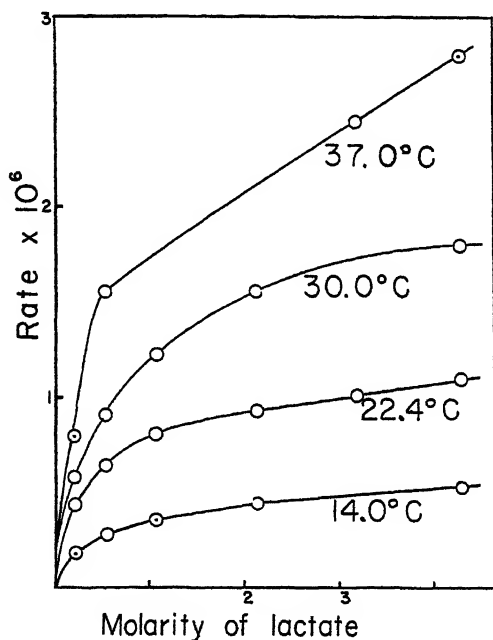


FIG. 2. Initial rates (moles/l./sec.) plotted against lactate concentration, at a series of temperatures and using the dye phenosafranine. Optimal concentrations of enzyme and coenzyme were used.

coenzyme concentration. The rates are seen to obey the usual Michaelis-Menten type of law.

In Fig. 3 the rate is plotted against the concentration of coenzyme, and it is seen that the rate now passes through a maximum and decreases at higher concentrations. This result is different from that obtained previously; both Green and Brosteaux (2) and Straub (5) report

that there is a leveling-off of the rate at high coenzyme concentrations. The difference is due to our use of coenzyme concentrations about 10 times as high as those employed previously.

Initial rates are plotted against enzyme concentration in Fig. 4. The enzyme concentration is indicated as milliliters of enzyme extract used, since, the molecular weight being unknown, the molar concentration cannot be obtained. In agreement with Straub's result the rate is seen to pass through a maximum.

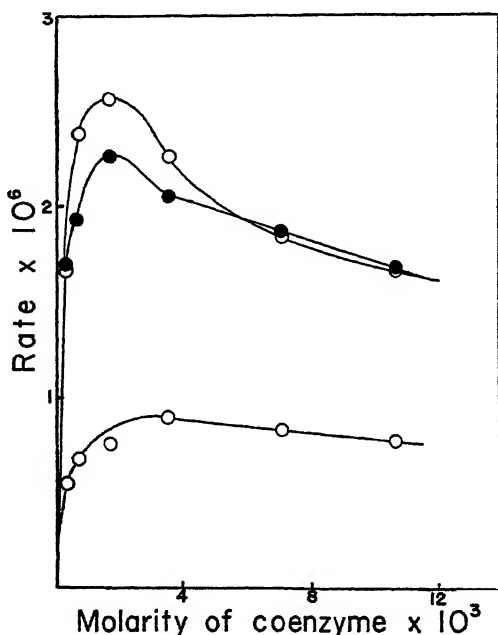


Fig. 3. Initial rates (moles/l./sec.) plotted against coenzyme concentration, corresponding to different amounts of enzyme and lactate;  $T = 37^\circ\text{C}$ .

Typical Arrhenius plots of  $\log k$  vs.  $1/T$  are shown in Fig. 5. The activation energies obtained are summarized in Table I. These values were obtained with the object of determining the separate influences of (a) the enzyme concentration, (b) the lactate concentration, and (c) the coenzyme concentration, on the energy of activation. By low concentration in the table is implied a concentration corresponding to the linear part of the appropriate rate vs. concentration curve; by opti-

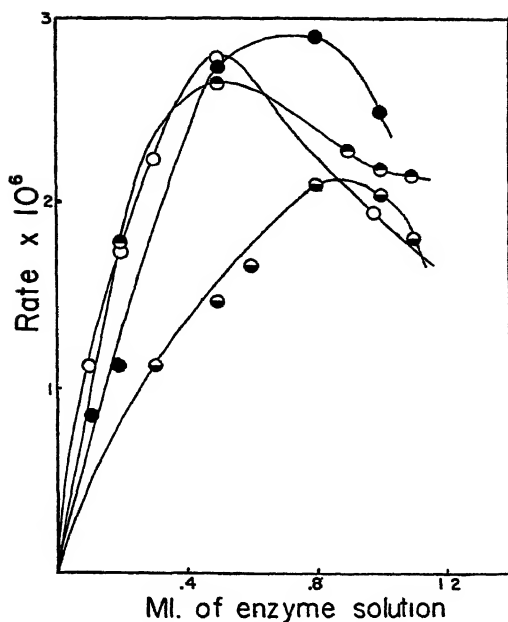


FIG. 4. Initial rates (moles/l./sec.) plotted against enzyme concentration, expressed in ml. of extract used.

TABLE I  
Summary of Activation Energies

Concentration			$k \times 10^3$ at 30°C.	$E$
Enzyme	Coenzyme	Lactate		
Low	Low	0	—	$\sim 5$ (extrap.)
Low	Low	0.107	0.239	$6.7 \pm .2$
Low	Low	0.124	0.159	$8.7 \pm .2$
Low	Low	0.430	0.116	$12.2 \pm .3$
Low	Low	$\infty$	—	$\sim 13$ (extrap.)
Optimal	Low	0.107	1.07	$11.4 \pm .3$
Optimal	Low	0.214	0.615	$12.0 \pm .3$
Optimal	Low	0.430	0.348	$12.2 \pm .3$
Low	Optimal	0	—	$\sim 11$ (extrap.)
Low	Optimal	0.107	0.678	$10.8 \pm .3$
Low	Optimal	0.214	0.445	$12.5 \pm .4$
Low	Optimal	0.430	0.228	$11.8 \pm .3$

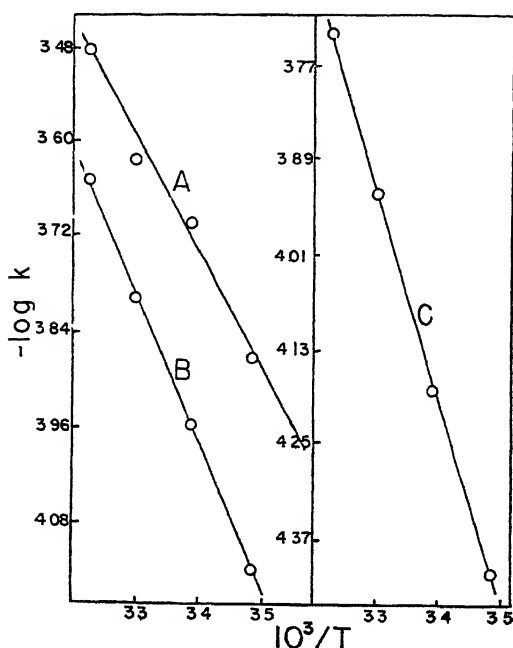


FIG. 5. Plots of  $\log k$  vs.  $1/T$  for three different lactate concentrations. (A) 0.017 M, (B) 0.214 M, (C) 0.430 M.

mal concentration, one corresponding to the maximum rate. All of the values quoted were obtained using phenosafranine as the dye; results were also obtained using methylene blue and are identical within the experimental error. The activation energies and the errors were obtained from the data using the method of least squares.

### DISCUSSION

The kinetics of enzyme-catalyzed reactions involving a single substrate and no coenzyme can for the most part be explained on the basis of the assumption of a binary complex between enzyme and substrate. The relationship between rate and substrate concentration was first derived by Michaelis and Menten (10, 11), and a more general treatment, which includes the effect of enzyme concentration, has been developed recently (12). According to these treatments, the rate of reaction rises at first linearly with increasing concentration of either

enzyme or substrate, but eventually becomes independent of the concentration; the analogy with surface reactions is very close. A modification of the treatment is required in the case of the urease-catalyzed hydrolysis of urea, in which the water must be regarded as a second substrate (13, 14).

With systems involving a substrate and a coenzyme, such as the one under consideration, the situation is considerably more complicated; some of the possible types of behavior have recently been discussed (6). There is now a ternary complex formed, and this can be envisaged as involving the "adsorption" of a lactic acid molecule on one site on the enzyme molecule, and the simultaneous "adsorption" of a coenzyme molecule on a neighboring site; given certain energy and entropy requirements the complex may then pass over a free-energy barrier and give rise to the reaction products, by a transfer of two hydrogen atoms.

If these adsorption processes are of the noncompetitive type, *i.e.*, if lactic acid cannot be adsorbed on the second type of site, and coenzyme cannot be adsorbed on the first type, it can easily be shown (6) that the rate will obey a law of the Michaelis-Menten type with respect to both substrate and coenzyme. If, on the other hand, there is competitive adsorption, the lactic acid and coenzyme being adsorbed on both types of sites, the treatment shows that the rate will at first increase linearly with respect to either concentration, reach a maximum, and then diminish. Explicit expressions for these two cases have been developed. In the present system it has been seen that the rate reaches a limiting value with respect to lactic acid concentration, but reaches a maximum and then diminishes with respect to coenzyme concentration. These facts suggest that the coenzyme can be adsorbed not only on its own type of site but also on the lactic acid type; the lactic acid molecules, on the other hand, can only be adsorbed on sites of the one type. The kinetic law pertaining to this system will now be developed.

### *Kinetic Law*

For reaction to occur it is postulated that a lactic acid molecule  $L$  must be adsorbed on a site of Type 1, and a coenzyme molecule  $C$  on a site of Type 2. Adsorption of  $C$  on a site of Type 1 is also possible, but does not contribute to the reaction.

Let  $\theta_1$  be the fraction of sites of Type 1 covered by  $L$ , and  $K_1$  be the corresponding equilibrium constant; similarly let  $\theta_2$  and  $K_2$  relate to  $C$  on sites of Type 2. Let  $\theta_1'$  be the fraction of sites of Type 1 covered by  $C$ , and  $K_1'$  the corresponding equilibrium constant.

For the sites of Type 1 the fraction covered by  $L$  is  $\theta_1$ , and the fraction bare is  $1 - \theta_1 - \theta_1'$ . The equilibrium involving  $L$  and sites of Type 1 is therefore given by

$$\frac{\theta_1}{1 - \theta_1 - \theta_1'} = K_1[L], \quad [1]$$

where  $[L]$  is the concentration of free lactic acid present. This concentration is related to the concentration of lactic acid introduced  $[L]_0$ , by

$$[L] = [L]_0 - \theta_1[E]_0, \quad [2]$$

since  $\theta_1[E]_0$  is the concentration of enzyme-lactic acid complexes. Hence

$$1 - \theta_1 - \theta_1' = K_1\{[L]_0 - \theta_1[E]_0\}. \quad [3]$$

The equilibrium involving  $C$  and sites of Type 2 is similarly

$$\frac{\theta_2}{1 - \theta_2} = K_2[C] \quad [4]$$

$$= K_2\{[C]_0 - [E]_0(\theta_2 + \theta_1')\}, \quad [5]$$

and that involving  $C$  and sites of Type 1 is

$$\frac{\theta_1'}{1 - \theta_1 - \theta_1'} = K_1'[C] \quad [6]$$

$$= K_1'\{[C]_0 - [E]_0(\theta_2 + \theta_1')\}. \quad [7]$$

Solution of Eqs. [3], [5], and [7] gives approximately

$$\theta_1 = \frac{K_1[L]_0}{1 + (K_1 + K_1')[E]_0 + K_1[L]_0 + K_1'[C]_0} \quad [8]$$

and

$$\theta_2 = \frac{K_2[C]_0}{1 + K_2[E]_0 + K_2[C]_0}. \quad [9]$$

The concentration of ternary complexes of the type that give rise to reaction, *i.e.*, having  $L$  on site of Type 1 and  $C$  on site of Type 2, is  $[E]_0\theta_1\theta_2$ , and the rate of reaction is therefore  $k_0[E]_0\theta_1\theta_2$ , where  $k_0$  is the rate constant for the decomposition of the complex; hence the reaction rate is

$$v = \frac{k_0 K_1 K_2 [E]_0 [L]_0 [C]_0}{\{1 + (K_1 + K_1')[E]_0 + K_1[L]_0 + K_1'[C]_0\} \{1 + K_2[E]_0 + K_2[C]_0\}}. \quad [10]$$

This expression may be seen to predict qualitatively the type of behavior observed in the present investigation. Thus at low lactic acid concentrations  $K_1[L]_0 \ll 1 + (K_1 + K_1')[E]_0 + K_1'[C]_0$ , and the rate is therefore directly proportional to the lactic acid concentrations; at high concentrations, however,

$$K_1[L]_0 \gg 1 + (K_1 + K_1')[E]_0 + K_1'[C]_0,$$

so that the rate becomes independent of the lactic acid concentration. The expression therefore accounts for the behavior represented in Fig. 2. With increasing coenzyme concentration, on the other hand, the

rate is seen to pass through a maximum, since at high values of  $[C]_0$  the rate is inversely proportional to  $[C]_0$ ; this conclusion is consistent with Fig. 3. Similarly the mechanism predicts a maximum with increasing enzyme concentration, as in Fig. 4.

### Heats of Reaction and Activation

The experimental data can be partially analyzed with reference to Eq. 10, to give information regarding the heats of reaction and of activation. Let the heat of activation corresponding to the reaction of the ternary complex, *i.e.*, corresponding to the rate constant  $k_0$ , be denoted as  $\Delta H_0^*$ , and let the heats associated with the equilibrium constants  $K_1$ ,  $K_2$ , and  $K_1'$  be respectively  $\Delta H_1$ ,  $\Delta H_2$ , and  $\Delta H_1'$ . At very low concentrations of enzyme, lactic acid, and coenzyme, the rate is seen from Eq. 10 to be given by

$$v = k_0 K_1 K_2 [E]_0 [L]_0 [C]_0 \quad [11]$$

and the experimental activation energy is therefore equal to  $\Delta H_0^* + \Delta H_1 + \Delta H_2$ . At low concentrations of enzyme and coenzyme but high concentrations of lactic acid, the rate is given by

$$v = k_0 K_2 [E]_0 [C]_0, \quad [12]$$

and the experimental activation energy is therefore  $\Delta H_0^* + \Delta H_2$ . The rate corresponding to low concentrations of enzyme and lactic acid and to the optimal concentration of coenzyme is obtained by setting  $dv/d[C]_0$  equal to zero; this gives  $[C]_0(\text{max.}) = 1/(K_1' K_2)^{1/2}$ , and thence leads to

$$v = \frac{k_0 K_1 K_2^{1/2}}{K_1'^{1/2}} [E]_0 [L]_0. \quad [13]$$

The experimental activation energy is thus  $E = \Delta H_0^* + \Delta H_1 + \frac{1}{2}\Delta H_2 - \frac{1}{2}\Delta H_1'$ . These results are collected in Table II, which also gives the relevant experimental activation energies. From the values the following can be derived

$$\begin{aligned} \Delta H_1 &= -8 \text{ kcal.} \\ \Delta H_2 + \Delta H_1' &= -12 \text{ kcal.} \end{aligned}$$

TABLE II  
Analysis of Activation Energies

Concentration				<i>E</i>
Enzyme	Lactate	Coenzyme		
Low	Low	Low	$\Delta H_0^* + \Delta H_1 + \Delta H_2$	kcal. 5
Low	High	Low	$\Delta H_0^* + \Delta H_2$	13
Low	Low	Optimal	$\Delta H_0^* + \Delta H_1 + \frac{1}{2}\Delta H_2 - \frac{1}{2}\Delta H_1'$	11

These energy relationships are represented schematically in Fig. 6, in which the enzyme molecule is denoted by  ${}_1E_2$ , the subscripts representing the sites of Types 1 and 2. When a lactic acid molecule becomes attached to site of Type 1 on the enzyme there is a decrease in  $H$  of 8 kcal. (i.e.,  $\Delta H_1 = -8$  kcal.), 8 kcal. of heat being liberated. When in addition the coenzyme molecule becomes attached there is presumably a further liberation of heat, but the amount is unknown; it is represented as  $x$  kcal. in the diagram. The activation energy for the reaction of the ternary complex  $L-{}_1E_2-C$  is seen to be  $13 + x$  kcal. The increase in activation energy with increasing lactate and coenzyme

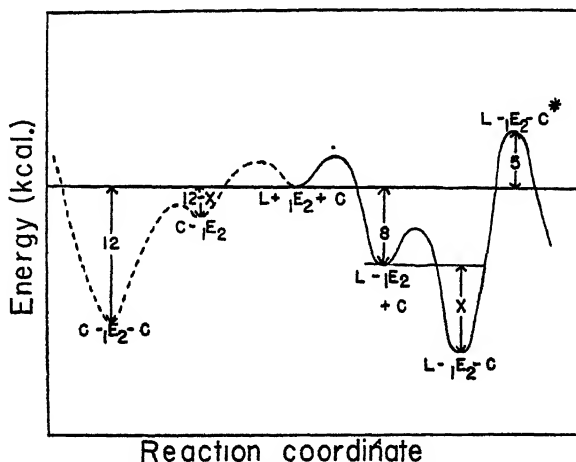
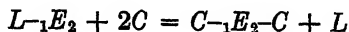


FIG. 6. Schematic representation of energy levels for binary and ternary complexes.

concentrations is due to the fact that at low concentrations the complexes are largely dissociated, so that the activation energy approaches 5 kcal.; at high concentrations, however, the system is largely in the form of complex, and the activation energy approaches  $13 + x$  kcal. It may be noted that the fact that the activation energy increases with increasing concentrations of lactate and coenzyme is evidence that the binary and ternary complexes are formed exothermically.

The energy levels for the complexes involving the adsorption of coenzyme on the lactic acid sites are also shown in Fig. 6 (dotted curve). The binary complex  $C-{}_1E_2$  is formed with the evolution of an amount of heat  $12 - x$  kcal., while the ternary complex  $C-{}_1E_2-C$  is formed from

$2C + {}_1E_2$  with the evolution of 12 kcal. This ternary complex is more stable than the binary complex  $L-{}_1E_2$  by 4 kcal., which means that the replacement reaction



is exothermic by 4 kcal. This is related to the strong inhibiting action of the coenzyme at high concentrations.

The data also permit a value to be obtained for the entropy change associated with the formation of the binary complex  $L-{}_1E_2$ . From the variation of the rate with the lactic acid concentration, a value of  $K_1 = 20$  l./mole at 300°K. can be deduced, and this corresponds to a free-energy change  $\Delta F_1$  of  $-1.78$  kcal. With the value  $\Delta H_1 = -8$  kcal., this leads to an entropy decrease of about 20 entropy units when the complex is formed. This is a reasonable value for a bimolecular association of this kind.

The present result, that the complexes are formed exothermically and with an entropy decrease, is to be contrasted with the evidence recently obtained for reactions catalyzed by urease (13, 14) and by pepsin (15) that the complexes are formed endothermically and that the process is accompanied by an increase in entropy. Such effects may only be associated with enzymes which are concerned with the performance of mechanical (*e.g.*, osmotic) work in living systems, and are therefore perhaps not to be expected with the hydrogenases.

The authors wish to thank Dr. D. E. Green for valuable suggestions relating to this work.

### SUMMARY

1. The kinetics of the autoxidation of lactic acid have been investigated under conditions which ensure that the rate-controlling step is



this reaction requiring the apoenzyme of lactic dehydrogenase.

2. The rate increases at first linearly with increasing concentrations of enzyme, lactic acid, and coenzyme. At higher concentrations of lactic acid, the rate reaches a constant limiting value, while at higher concentrations of either enzyme or coenzyme the rate passes through a maximum and then decreases.

3. The activation energy of the reaction increases markedly with increasing concentration of lactic acid and of coenzyme.

4. The results are shown to be consistent with an enzyme model involving the assumption of two adsorption sites, Types 1 and 2, on the enzyme molecule. For reaction to occur a lactic acid molecule must be adsorbed on a site of Type 1, and a coenzyme molecule on a site to Type 2, and certain energy and entropy requirements must be satisfied. It is supposed that coenzyme molecule can also be attached to a site of Type 1, replacing a lactic acid molecule and so accounting for the inhibition at high coenzyme concentrations.

5. The activation energies obtained are analyzed in terms of the model proposed, and some conclusions are drawn about heat and entropy changes involved in the formation of the various binary and ternary complexes.

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# The Mechanism of Resistance to Sulfonamides. VI. A Comparative Study of the Resistance to Sulfathiazole of the Metabolism of Glucose and Pyruvate by *Staphylococcus aureus*<sup>1</sup>

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## INTRODUCTION

In a previous study by Sevag and Green (1), it was reported that the growth of a sulfonamide resistant strain of *Staphylococcus aureus* in casein hydrolyzate and glucose was resistant to 0.04 *M* sulfanilamide (SA), or 0.0066 *M* sulfathiazole (ST). The growth of this strain was inhibited up to 75% when pyruvate substituted glucose. It was then stated that "With the development of resistance in a medium containing glucose there develops a highly specialized *sulfonamide-resistant* mechanism for the metabolism of glucose." On the basis of the above results, Work and Work (2) implied that "such a result suggested that sulfonamides blocked some step in the normal metabolism of glucose through pyruvate, and that the resistant organism developed an alternate metabolic process which by-passed pyruvate." However, in the light of the results reported herein and referred to below, it has been felt that the above statements require amplification. Results from this laboratory (3, 4) showed that there are basic differences in the interrelationship of the amino acid requirements and glucose metabolism by the susceptible and resistant strains of *Staph. aureus*. Also, the results from this and other laboratories show that sulfonamides block the tricarboxylic acid cycle via pyruvate, succinate,

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glutamic acid, etc. Since the tricarboxylic acid cycle is the pathway for the formation of  $\alpha$ -keto acids, amino acids and, therefore, of proteins, it is necessary that we have clear information as to whether or not the pyruvate metabolism by the resistant cells is resistant or susceptible to sulfonamides. In view of these interrelationships the question of the relative resistance of pyruvate metabolism to sulfathiazole during growth was therefore reinvestigated. In the previous study (1), relatively high concentrations of sulfonamides, 6880  $\mu\text{g.}$  of SA/ml. (0.0066  $M$ ) were used. Under these conditions, the relative insensitivity of pyruvate metabolism to sulfonamides by the resistant cells was apparently masked. Using 250  $\mu\text{g.}$  of ST/ml. medium, the growths of three pairs of susceptible and resistant strains of *Staph. aureus* in media containing glucose or pyruvate, with or without tryptophan, were determined. From the tabulated results it can be seen that the growth of the resistant strains in a medium containing pyruvate is just as resistant to sulfathiazole as the growth in media containing glucose. Thus, the original theory of a specialized sulfonamide resistant mechanism for the metabolism of glucose is extended to include also the steps involving pyruvate metabolism which previously was not part of the theory for the reasons stated above.

### EXPERIMENTAL METHODS

The strains of *Staphylococcus aureus*, the growth medium, and the method used for the determination of the weight of cells were the same as those used previously (3, 4).

Glucose was determined according to Folin and Wu (5), and pyruvate according to Bueding and Wortis (6).

### DISCUSSION OF RESULTS

The results presented in Table I show that the growths of three *sulfonamide-susceptible* strains, 1A, 2A, and 3A, in the presence of *glucose*, with or without tryptophan, are inhibited from 74 to 100%. These inhibitions are associated with from 23 to 100% inhibition of glucose metabolism.<sup>2</sup> With the exception of the resistant strain 1E,

<sup>2</sup> The data presented in Table I show that glucose does not disappear when the growth of the strains 2A and 3A are completely inhibited by sulfathiazole. With the strain 2A when pantothenate is added to the medium, 90% inhibition of growth is associated with 49% inhibition of glucose disappearance (data not reported here). As will be seen in Table I, the 74 to 91% inhibition of the growth of strain 1A is associated with 23 to 36% inhibition of glucose utilization. These data may indicate that under conditions of partial growth inhibition, there can occur inhibition of

TABLE I

*Comparison of the Susceptibility and Resistance to Sulfathiazole of the Growth in Casein Hydrolyzate-Glucose Medium of Three Pairs of Susceptible and Resistant Strains of Staphylococcus aureus*<sup>a</sup>

Strains	Basal medium + 0.5% glucose								
	Tryptophan					No tryptophan			
	µg. of ST/ml.	Mg. of cells/ 10 ml.	Mg. of glucose used/ mg. cells	% inhibition		Mg. of cells/ 10 ml.	Mg. of glucose used/ mg. cells	% inhibition	
				Growth	Glucose used			Growth	Glucose used
1A (Susceptible)	0 250	2.93 0.77	19.2 14.8	— 74	— 23	2.38 0.21	21.9 14.0	— 91	— 36
1E (Resistant)	0 250	2.39 1.11	21.9 44.8	— 53	— -104	2.00 1.38	14.7 30.0	— 31	— -104
2A (Susceptible)	0 250	4.91 0.5	11.8 0	— 90	— 100	4.64 0	11.7 0	— 100	— 100 <sup>b</sup>
2E (Resistant)	0 250	3.49 3.46	15.1 14.5	— 0	— 4	3.52 3.26	14.2 14.2	— 7	— 0
3A (Susceptible)	0 250	2.53 0	20.8 0	— 100	— 100 <sup>b</sup>	2.83 0	17.6 0	— 100	— 100 <sup>b</sup>
3E (Resistant)	0 250	3.10 2.35	15.4 18.7	— 24	— -21	2.58 1.88	16.8 23.0	— 27	— -43

*Inoculum.* A second transplant on extract agar which was grown for 18 hr. was washed twice with phosphate buffer. 250,000 cells from the washed suspension were used as inoculum/ml. of medium in the systems presented in Tables I and II.

*Growth period.* Was 44 hr. at 37°C.

<sup>a</sup> The data are representative of simultaneously performed quadruplet test systems.

<sup>b</sup> For a consideration of these values see footnote 2.

the corresponding *resistant strains*, 2E and 3E, are resistant to sulfathiazole under the same conditions. This resistance is likewise associated with a glucose metabolism free from inhibition.

glucose used/mg. cells. However, according to the results reported before (4), sulfathiazole accelerates the breakdown of glucose but blocks the utilization of the breakdown products for growth purposes. The latter effect may appear to represent a more critical feature of sulfonamide action under growth conditions.

In the presence of pyruvate (Table II) the growths of the *susceptible* strains, 1A, 2A, and 3A, are likewise highly susceptible to sulfathiazole. Unlike the inhibition of glucose metabolism during growth by these strains, the inhibition of the growth in pyruvate-containing medium is associated with an accelerated pyruvate metabolism (25 to 146%) in the presence of tryptophan. In contrast, in the absence of tryptophan, 40–87% inhibition of the growth of the strains 2A and 3A is associated with 30–40% inhibition of pyruvate metabolism. (Strain 1A possesses strain characteristics which will be discussed below.)

TABLE II

*Comparison of the Susceptibility and Resistance to Sulfathiazole of the Growth in Casein Hydrolyzate-Pyruvate Medium of Three Pairs of Susceptible and Resistant Strains of Staphylococcus aureus*<sup>a</sup>

Strains	Basal medium + 0.5% pyruvate								
	Tryptophan					No tryptophan			
	$\mu\text{g}$ of ST/ml.	Mg. of cells/10 ml.	Mg. pyruvate used/mg. cells	% inhibition		Mg. of cells/10 ml.	Mg. pyruvate used/mg. cells	% inhibition	
				Growth	Pyruvate used			Growth	Pyruvate used
1A (Susceptible)	0 250	2.70 1.10	8.5 20.9	— 60	— -146	2.13 0.60	11.0 38.8	— 72	— -253
1E (Resistant)	0 250	0.83 2.65	28.1 8.9	— -220	— 68	1.64 2.38	14.1 9.7	— -45	— 31
2A (Susceptible)	0 250	>1.43 <sup>b</sup> 0.83	16.0 <sup>b</sup> 26.6	— >42	— -66	0.64 0.39	27.0 16.9	— 40	— 40
2E (Resistant)	0 250	1.68 1.52	13.7 15.2	— 9	— -10	1.45 1.43	15.8 16.1	— 0	— 0
3A (Susceptible)	0 250	1.56 0.37	14.7 18.4	— 76	— -25	1.38 0.18	16.7 11.6	— 87	— 30
3E (Resistant)	0 250	2.23 1.90	10.2 12.0	— 14	— -18	2.08 1.68	10.9 13.4	— 20	— -23

<sup>a</sup> The data are representative of simultaneously performed quadruplet test systems.

<sup>b</sup> Due to slight clumping of the strain 2A in these growth systems, the indicated values represent close approximations only.

Apparently, the presence of tryptophan accelerates, and its absence causes, the inhibition of pyruvate metabolism in the presence of sulfathiazole. The growths of the corresponding *resistant strains*, 2E and 3E, are resistant to sulfathiazole. Here the acceleration of pyruvate metabolism by sulfathiazole in the presence of tryptophan is markedly reduced, and, in the absence of tryptophan it is associated either with a negligible acceleration, or is free from inhibition. These relationships are, therefore, the reverse of those pertaining to the corresponding susceptible strains.

The data appear to show a certain as yet undefined interrelated pyruvate-tryptophan metabolism in the mechanism of resistance.

#### *Strain Characteristics of 1E and 1A*

The resistant strain 1E, unlike the resistant strains 2E and 3E, as discussed elsewhere (1, 3), is more resistant to sulfonamides in the absence of *glucose*. The growth of this strain is inhibited (Table I) in the presence of glucose from 31 to 53% (in the absence and presence of tryptophan, respectively) despite the fact that sulfathiazole causes 104% accelerated breakdown of glucose. It thus appears that this strain is unable to utilize fully for growth purposes the ST-accelerated breakdown products of glucose.

In media containing *pyruvate*, the strain 1E represents a reverse picture. Here, growth in the presence of sulfathiazole is accelerated 220% (in the presence of tryptophan) and 45% (in the absence of tryptophan) despite the fact that these accelerations are associated with a 68% and 31% inhibition of pyruvate metabolism respectively. In contrast, the growth of the susceptible strain 1A is inhibited from 60 to 72% by sulfathiazole despite the fact that the pyruvate metabolism is accelerated from 146% (tryptophan present) to 253% (tryptophan absent) by the drug. These data also show that this strain is unable to utilize for growth purposes the ST-accelerated pyruvate metabolic products.

#### SUMMARY AND CONCLUSIONS

The growths of three pairs of sulfonamide-susceptible and sulfonamide-resistant strains of *Staphylococcus aureus* in casein hydrolyzate containing glucose or pyruvate, with and without tryptophan, are compared. The simultaneous utilization of glucose and pyruvate in the

presence and absence of sulfathiazole are also compared. The differences characteristic of susceptible and resistant strains are discussed. An interrelated tryptophan-pyruvate metabolism has been indicated. The observations presented here show that growths of the resistant strains in glucose or pyruvate are resistant to sulfonamides, and, therefore, involve a resistant type of glucose and pyruvate metabolism. They do not appear to suggest that the resistant organisms by-pass pyruvate metabolism through an alternate metabolic process. Resistance to sulfonamides of the growth which is promoted several fold by glucose and pyruvate metabolism requires that key steps in the tricarboxylic acid cycle via pyruvate be also resistant to sulfonamides. The data pertaining to this question are discussed in the literature cited (3, 4).

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# The Estimation of Glycogen with the Anthrone Reagent <sup>1</sup>

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## INTRODUCTION

Most of the methods currently used for the determination of glycogen in tissues are based upon the procedure described by Pflüger (1) in 1905. This method consists of the digestion of the tissue in hot concentrated KOH solution, precipitation of the glycogen with ethanol, hydrolysis of the precipitate with mineral acid, and determination of the glucose in the hydrolyzate as reducing sugar. A major advance in the application of the method has been made by Good, Kramer and Somogyi (2), who have altered the conditions described by Pflüger so that an analysis can be carried out in several hours instead of 2 days as required by the original procedure. Other proposed methods depend upon the measurement of the color produced by glycogen with iodine (3), and with certain organic reagents such as diphenylamine (4).

The use of one such organic reagent, anthrone, was first described by Dreywood (5) as a qualitative test for carbohydrates. Subsequently, Morris (6) adapted the use of the anthrone reagent to the quantitative estimation of carbohydrates, and suggested its use for the determination of glycogen. The mechanism of the reaction between anthrone and carbohydrates in general was studied by Sattler and Zerban (7), who postulated that the sulfuric acid medium of the reagent causes dehydration of the sugar to a furfural derivative which then presumably condenses with anthrone to form a blue-colored compound.

The purposes of the present paper are: (a) to present conditions for making the quantitative determination of certain carbohydrates by the anthrone reagent simple and reproducible, eliminating the vagaries

<sup>1</sup> A preliminary report of this work was presented before the American Society of Biological Chemists, Detroit, Michigan, April 20, 1949 [Seifter, S., and Dayton, S., *Federation Proc.*, 8, 249 (1949)].

of the reaction previously noted by Morris; (b) to describe several procedures for the use of the anthrone reagent in the determination of glycogen in tissues of high and low glycogen contents respectively; (c) to compare results obtained in the analysis of tissue glycogen with the anthrone reagent with those obtained by the method of Good, Kramer and Somogyi; and (d) to describe the extent of interference engendered by the presence of proteins and protein hydrolytic products in carbohydrate solutions being reacted with the anthrone reagent.

### MATERIALS AND EQUIPMENT

1. 30% potassium hydroxide solution.
2. 95% ethanol.
3. 60% ethanol.
4. 95% sulfuric acid. This is prepared by adding 1 l. of the best grade concentrated sulfuric acid to 50 ml. of distilled water and cooling the resultant solution.
5. 0.2% anthrone solution. This constitutes the working reagent, and is made by dissolving 0.2 g. of anthrone<sup>2</sup> in 100 ml. of 95% sulfuric acid. The reagent is not stable in solution for extended periods of time, and is best made up daily as needed (8).
6. A working glucose standard solution containing 20  $\mu$ g. of glucose/ml.
7. A photoelectric colorimeter. The work reported here was carried out using an Evelyn macro photoelectric colorimeter; however, other similar instruments of different manufacture may be used if suitable adaptations are made in the methods described.

### CONDITIONS FOR THE ANTHRONE-CARBOHYDRATE REACTION

In the course of preliminary work using the procedure described by Morris (6), it was observed that considerable discrepancies often occurred among results obtained on identical samples even when pure glucose solutions were used. This was traced to the fact that this procedure relies upon the heat generated by the addition of the anthrone reagent to the aqueous test solution. Although tubes of uniform size were used, as suggested by Morris, the rates of heat liberation and dissipation appeared to vary significantly. This difficulty was overcome by adding the reagent to the test solutions while the tubes were submerged in a bath filled with cold water, subsequently heating the tubes in a boiling water-bath, and finally cooling them in cold water. When this procedure is followed, uniform tubes are not required, the only

<sup>2</sup> Anthrone is easily prepared from anthraquinone according to the directions given in *Org. Syntheses* 1, 52 (1932). An adequate summary of the synthesis is given by Morris (6).

precaution being that *complete* mixing of the reagent and the test solutions must be obtained while keeping the tubes bathed in cold water. This even mixing can be achieved by rapidly swirling the tubes in the water-bath. When all of the above conditions are met, the curve of color development at 620  $m\mu$  reaches a maximum after 9–10 min. of heating, while more prolonged heating causes the depth of color to fall off at a considerable rate. A 10-min. heating period has accordingly been adopted for all determinations with the anthrone reagent.

The color so developed follows Beer's law, and is stable for several hours at room temperature. Moreover, if the depth of color produced by a given sample is too great to permit a reading to be made on the colorimeter, a second reading may be made, without introducing too much error in the determination, after adding an appropriate volume of a cold solution containing 1 part distilled water and 2 parts anthrone reagent.

Inasmuch as the reagent deteriorates on standing, a standardization curve may be employed only if the reagent is prepared on the day of its intended use. With freshly prepared anthrone solutions the colorimeter reading obtained for the standard is reasonably constant.

An outline of the procedure finally adopted for the determination of carbohydrate in a test solution follows:

- a) To the bottom of an Evelyn macro colorimeter tube are delivered 5 ml. of the test solution containing an amount of carbohydrate equivalent to 15–150  $\mu g.$  of glucose.
- b) To a second Evelyn tube are added 5 ml. of the working glucose standard containing a total of 100  $\mu g.$  of glucose.
- c) To a third tube are added 5 ml. of distilled water; this constitutes the blank.
- d) While submerged in cold water each tube then receives 10 ml. of the anthrone reagent delivered from a fast-flowing pipet or buret, and the reactants are mixed by swirling the tubes.
- e) The cold tubes are then covered with glass marbles and heated for 10 min. in a boiling water-bath.
- f) The tubes are then immediately cooled in a bath containing cold water, and read in the colorimeter at 620  $m\mu$  after the galvanometer has been set at 100 with the blank.

Calculations can be made using a modified colorimetric formula as will be described under the determination of glycogen.

## DETERMINATION OF GLYCOGEN IN TISSUES

### a) *A Direct Method for Liver Glycogen*

Immediately after excision from the animal, approximately 1 g. of liver is dropped into a previously weighed test tube containing 3 ml. of 30% KOH solution. After

delivery of the sample, the tube and contents are reweighed and the weight of sample determined by difference. The tissue is then digested by heating the tube for 20 min. in a boiling water-bath, and following this the digest is cooled, transferred quantitatively to a 50-ml. volumetric flask, and diluted to the mark with water. The contents of the flask are thoroughly mixed, and a measured portion is then further diluted with water in a second volumetric flask so as to yield a solution of glycogen concentration of 3-30  $\mu\text{g./ml.}$  Five-ml. aliquots of the final dilution are then pipetted into Evelyn tubes and the determination with anthrone is carried out as previously described. The amount of glycogen in the aliquot used is then calculated using the following equation:

$$\mu\text{g. of glycogen in aliquot} = \frac{100 \times U}{1.11 \times S},$$

$U$  = the optical density of the unknown test solution,

$S$  = the optical density of the 100- $\mu\text{g.}$  glucose standard,

1.11 = the factor determined by Morris (6) for the conversion of glucose to glycogen.

TABLE I

*A Comparison of Liver Glycogen Contents as Determined by the Method of Good, Kramer and Somogyi and by the Direct Anthrone Procedure*

Glycogen by method of Good, Kramer and Somogyi (A) %	Glycogen by direct anthrone procedure (B) %	$\frac{B}{A} \times 100$
1.42	1.45	102.0
2.27	2.14	94.3
2.28	2.37	104.0
3.19	3.07	96.3
3.74	3.97	106.1
4.08	4.03	98.7
4.53	4.27	94.3
4.71	4.43	94.1
4.74	4.70	99.2
5.43	5.59	103.0
5.98	5.61	94.0
6.17	5.85	94.8
6.39	6.75	105.6
6.78	7.05	104.0
6.84	6.82	99.7
7.62	7.70	101.0
8.50	8.17	96.2
8.51	8.44	99.2
8.72	8.55	98.1
Average		99.0
Standard deviation		$\pm 4.0$

The results obtainable by this method are illustrated in Table I, in which glycogen values determined on rat liver digests by this procedure are compared with results obtained on the same material by the method of Good, Kramer and Somogyi (2). The data show that comparable results are obtained by the two procedures for rat livers varying in glycogen content from approximately 1.5–9%. However, with glycogen contents lower than 1%, errors due to protein interference occur in the direct anthrone method as will be discussed below.

The direct procedure is applicable for the determination of glycogen in tissues of relatively high glycogen contents, and is therefore especially useful in nutritional and endocrinological experimentation requiring the assay of liver glycogen. The method has the advantages of brevity and simplicity as compared with other procedures, requiring little more than an hour for performance since it eliminates the necessity of both glycogen precipitation and hydrolysis, and requiring few and simply prepared reagents.

#### *b) An Indirect Method for Glycogen in Tissues of Low Glycogen Contents*

A snip of tissue weighing between 25 and 100 mg. is weighed on a micro torsion balance, placed in 1 ml. of 30% KOH solution contained in a tube suitable for subsequent centrifugation, and digested in a boiling water-bath for 20 min. The tube is then cooled, 1.25 ml. of 95% ethanol is added, and the contents mixed. (If a stirring rod is used for mixing, it should be washed with a small quantity of 60% ethanol.) The contents of the tube are gently brought to a boil in a hot water-bath, again cooled, and centrifuged for 15 min. at 3000 r. p. m. The supernate is decanted and the tube is allowed to drain on filter paper for a minute or two. Although not absolutely necessary, the precipitate may be redissolved in 1 ml. of distilled water and reprecipitated with 1.25 ml. of 95% ethanol. In either case, the sedimented glycogen, after drainage, is dissolved in exactly 5 ml. of water, and reacted with the anthrone reagent as described previously. The reacted mixture is then transferred to an appropriate colorimeter tube and read in the colorimeter. The glycogen content can then be calculated with the aid of the equation given in the discussion of the direct method.

The indirect procedure is recommended for the determination of glycogen in tissues of relatively low glycogen content such as diaphragm, skeletal muscle, heart muscle, and in certain instances, liver. Table II contains data showing the good agreement between results obtained by this procedure and by the method of Good, Kramer and Somogyi on various rat tissues. The agreement of results is better when tissue digests are used rather than tissue snips because of the greater uniformity of samples in the former case.

TABLE II

*A Comparison of the Glycogen Contents of Rat Tissue Digests and of Tissue Snips as Determined by the Method of Good, Kramer and Somogyi and by the Indirect Anthrone Procedure*

Tissue	Glycogen content by method of Good <i>et al.</i>	Glycogen content by indirect an- throne procedure
	Tissue digests μg. glycogen/ml. of digest	
Diaphragm	88.2	85.2
Diaphragm	68.4	69.8
Leg muscle	130.1	123.1
Leg muscle	144.5	145.0
Heart muscle	64.1	62.5
Heart muscle	79.4	77.2
Heart muscle	82.4	80.2
Heart muscle	85.5	84.9
	Tissue snips mg. of glycogen/100 g. tissue	
Diaphragm	108	113
Diaphragm	162	156
Diaphragm	243	276
Diaphragm	201	210
Diaphragm	236	255
Leg muscle	295	274
Leg muscle	139	168
Leg muscle	302	325

#### INTERFERENCE BY PROTEINS AND THEIR HYDROLYTIC PRODUCTS WITH THE ANTHRONE DETERMINATION OF GLYCOGEN

The principal error in the direct method for the determination of glycogen as applied to livers of normal and high glycogen contents is probably due to inaccuracies in sampling, inasmuch as the original alkaline digest may contain flocculent material which cannot always be dispersed evenly before aliquots are taken.<sup>3</sup> That this is the case is reflected in the figures given in Table I showing that the deviations

<sup>3</sup> The flocculent material which occasionally appears has been separated by centrifugation and studied further. It contains considerable amounts of a substance or substances which, even after 3 washings with 30% KOH, behaves like glycogen toward the anthrone reagent. Further, the washed material reacts with iodine in the same manner as glycogen. Therefore, in the direct anthrone procedure, the diluted liver digest cannot be clarified by centrifugation before aliquots are taken without the danger of introducing larger errors in the determination than the relatively minor errors occasioned by direct sampling.

between results obtained on the same liver digests by this method and by a standard glycogen method are not consistently in one direction, and in this particular series reveal a standard deviation of  $\pm 4.0$ . Further, since the protein to glycogen ratio is relatively small in these livers, the protein becomes sufficiently diluted so that error due to its presence is well within the larger error due to sampling. However, in tissues of low glycogen content analyzed by the direct procedure, the protein to glycogen ratio in the sample required is relatively large, and the interference becomes correspondingly more significant. Accordingly, it was thought of interest to determine the actual extent of interference by proteins and their hydrolytic products which may be incurred when the direct anthrone procedure is performed.

*a) The "Glycogen Equivalent" of Tissue Freed from Glycogen*

Glycogen-free tissue was prepared by digesting rat liver in KOH and removing the glycogen by precipitation with ethanol. The material was then centrifuged, and the glycogen-free supernate carried through an analysis for glycogen-simulating color by the anthrone procedure. From this result and the determined nitrogen content of the tissue it was calculated that 1 mg. of tissue nitrogen was equivalent, on the average, to 5.0  $\mu$ g. of glycogen. Since the liver usually contains approximately 30 mg. of nitrogen/g. tissue, the theoretical error due to protein interference with the direct anthrone procedure can be shown to be 1.5% for a liver containing 1% glycogen, 0.5% for a liver having 3% glycogen, etc. As is obvious, the error due to protein interference for livers of normal or high levels of glycogen is well within the error due to sampling, and for all practical purposes may be ignored.

*b) Hemoglobin*

Inasmuch as the samples of liver contain a variable amount of blood, the extent of interference by hemoglobin was studied. It was established that each mg. of a pure preparation of beef hemoglobin is equivalent to approximately 10  $\mu$ g. of glycogen with respect to color developed with anthrone. However, when this color-equivalent is considered on the basis of an average amount of blood contained in

excised liver, the error in the direct glycogen determination due to hemoglobin can rarely exceed 0.8  $\mu$ g.

### c) *Amino Acids*

Since tissues being analyzed for glycogen first undergo a degree of alkaline hydrolysis, it was thought of interest to measure the color developed in the reaction between amino acids and the anthrone reagent. Solutions of 19 crystalline amino acids, each in the amount occurring in a 10 mg. sample of typical animal protein, were tested individually and all together for color development with anthrone. None of the amino acids tested, except tryptophan, gave a visible color with the reagent, and none gave significant absorption at 620  $m\mu$ . However, the mixture of all 19 gave a color equivalent to approximately 6 $\mu$ g. of glycogen. The latter figure, which represents the extent of interference expected if all of the protein in a typical direct glycogen analysis were hydrolyzed to amino acids, assumes significance, as previously noted, only for low-glycogen tissues.

Tryptophan, the only amino acid to give a visible color with anthrone, yielded an orange-red solution which showed a maximum absorption at 515  $m\mu$ . The color was specific enough to serve as a qualitative test to distinguish tryptophan from other amino acids, and could be obtained using tryptophan in the free or combined form. Gelatin, significantly enough, yielded no color with the anthrone reagent. Undoubtedly the red color obtained by Morris (6) with certain proteins was due to the presence of tryptophan. The unique behavior of this amino acid is in keeping with its capacity to condense readily with a variety of carbonyl-containing substances.

### d) *Miscellaneous Tissue Components*

The representative peptides, leucylglycine, leucylglycylglycine, and benzoylglycylglycine, in amounts of 150  $\mu$ g., gave no visible color and very insignificant absorption at 620  $m\mu$  when tested with the anthrone reagent. The pyrimidine and purine bases, thymine, uracil, adenine, guanine, and xanthine, tested in amounts ranging from 1-5 mg., gave no interfering color. Commercial samples of pentosenucleic and deoxypentosenucleic acids, in amounts from 0.2-2 mg., gave a small degree of interference with the glycogen determination, both exhibiting

some color development with the anthrone reagent. An interesting observation was made in regard to pentosenucleic acid: it develops a color with anthrone best and immediately in the cold, and much of the color disappears when the reaction mixture is heated as in the glycogen procedure. The color developed in the cold, moreover, is proportional to the amount of pentosenucleic acid present, albeit somewhat unstable at room temperature.

On the basis of the above observations, protein apparently constitutes the principal interfering substance in the direct anthrone procedure, and thus invalidates the application of this method to tissues of low glycogen content. The use of protein-free filtrates was therefore next considered. In this connection it was found that filtrates obtained from neutralized tissue digests with 4% trichloroacetic acid gave glycogen values with the anthrone reagent approximately 10% higher than the values obtained on the same tissue with either the Good, Kramer and Somogyi method or the previously described indirect anthrone procedure. Under the conditions employed, filtrates made by using trichloroacetic acid at concentrations higher than 4%, zinc hydroxide, or cadmium hydroxide were found entirely unsuitable for the determination of glycogen with anthrone.

### SUMMARY AND CONCLUSIONS

1. In extension of the work of Morris (6), conditions are presented which make the quantitative determination of certain carbohydrates with Dreywood's anthrone reagent simple and reproducible.

2. A direct method for the determination of glycogen in tissues of relatively high glycogen content, *e.g.*, liver, is described. This consists of reacting the anthrone reagent under specific conditions with a diluted aliquot of an alkaline digest of the tissue.

3. An indirect method for the determination of glycogen in tissues of relatively low glycogen content, *e.g.*, skeletal muscle, diaphragm, heart muscle, and in certain instances, liver, is presented. This consists of the reaction of anthrone with the glycogen separated from an alkaline digest of the tissue by the use of ethanol.

4. The interference with the anthrone procedure occasioned by the presence of proteins, peptides, amino acids, and other tissue components is discussed.

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# The Effect of Methadone Isomers on the Respiration of Rat Brain Homogenates

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## INTRODUCTION

Recent investigations have shown that racemic methadone inhibits the oxidation of glucose (1), pyruvate (2), succinate (2, 3), and the anaerobic glycolysis (1, 4) of glucose in brain tissue. These results are in accord with the theory proposed by Quastel and his associates (5) that narcosis is due to inhibition of carbohydrate metabolism in the brain.

Pharmacological experiments and clinical evaluations have shown that the analgesic activity of racemic methadone is primarily due to the *l*-isomer and that the *d*-form is much less effective as an analgesic. Scott, Robbins and Chen (6) found the analgesic action of *l*-methadone was 7.5, 25, and 50 times greater than *d*-methadone in the rat, dog, and man, respectively. Jenny and Pfeiffer (7) found the following minimal analgesic doses, in milligrams/kilogram, for guinea pigs: *l*-methadone, 5; *dl*-methadone, 12.5; *d*-methadone, 20; *l*-isomethadone, 7.5; *dl*-isomethadone, 12.5; *d*-isomethadone, 30. Isbell and Eisenman (8) have shown that *l*-methadone relieves the symptoms of morphine abstinence and produces addiction in man whereas *d*-methadone produces neither of these effects. The object of the present investigation was to determine the effect of methadone derivatives on the respiration of fortified brain homogenates, to investigate the site of enzymatic inhibition and especially to determine whether or not the optical isomers of methadone and isomethadone would show a variation in *in vitro* inhibition similar to that reported for their *in vivo* activity as analgesics.

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By using fortified tissue homogenates it is possible to investigate the effect of drugs on respiration in the presence of substrates which require only certain parts of the respiratory enzymatic chain. The tissue is diluted to eliminate as far as possible endogenous activity due to oxidizable material in the homogenate. Then by the addition of the necessary water-soluble components a high degree of activity is obtained in the presence of specific substrates. It is thus possible to determine what part of the enzymatic chain is most affected by the drug. Using these methods the effect of methadone derivatives on the anaerobic glycolysis of glucose and on the aerobic oxidation of ascorbate, succinate, and glucose has been determined.

### METHODS

The drugs used in these experiments were the hydrochlorides of *d*-, *l*-, and *dl*-methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) and the hydrochlorides of *d*-, *l*-, and *dl*-isomethadone (6-dimethylamino-5-methyl-4,4-diphenyl-3-hexanone) supplied by Mallinckrodt Chemical Works. Tissue homogenates were prepared by grinding fresh whole rat brain in cold distilled water or buffer (9). The rate of carbon dioxide evolution or oxygen uptake by the homogenates was followed in Warburg manometers. After a 10-min. period for gassing and temperature equilibration the stopcocks were closed and the initial reading taken. The substrate and homogenate were then mixed and readings taken at 10-min. intervals for 60 min.

With minor modifications the method of Utter, Wood and Reiner (10) was used for anaerobic glycolysis, that of Reiner (11) for the oxidation of glucose, and that of Schneider and Potter (12) for the oxidation of succinate and ascorbate. The concentration of solutions used, the additions to the homogenate, and the activity of the preparations are shown in Table I. The method of Edwards (13) was used to estimate the lactic acid in the reaction mixtures after some of the glycolytic experiments.

A Warburg apparatus with 14 manometers was used in order that duplicate or triplicate determinations could be made on control and experimental vessels containing homogenate from a single tissue preparation. In preliminary experiments the effect of a range of concentration of the isomers on oxidation of the substrates was investigated. Then simultaneous triplicate determinations were made on control vessels and on vessels containing the *d*-, *l*-, and *dl*- forms of methadone or isomethadone at a concentration which would give about 50% inhibition.

### RESULTS

Figure 1 shows the effect of *dl*-methadone on the oxidation of ascorbate by the rat brain homogenate. Oxygen uptake was linear with time. Inhibition of oxygen consumption by all the methadone derivatives at all concentrations was uniform for the 60-min. experiment.

TABLE I

*Effect of Methadone Derivatives on the Respiration of Brain Homogenates*

Each value is an average for 3 determinations. All experiments were of 60-min. duration at 36.3°C. Concentration of solutions: Ascorbate oxidation: sodium ascorbate, 0.0114 *M* from side arm; cytochrome *c*, 0.00013 *M*;  $\text{CaCl}_2$ , 0.0004 *M*;  $\text{AlCl}_3$ , 0.0004 *M*;  $\text{Na}_2\text{HPO}_4$ -HCl buffer, pH 7.4, 0.03 *M*; 0.3 ml. of 1:4 rat brain homogenate; final volume, 3.0 ml. Succinate oxidation: sodium succinate, 0.05 *M* from side arm; cytochrome *c*, 0.00013 *M*;  $\text{Na}_2\text{HPO}_4$ -HCl buffer, pH 7.4, 0.03 *M*;  $\text{AlCl}_3$ , 0.0004 *M*;  $\text{CaCl}_2$ , 0.0004 *M*; 0.3 ml. of 1:4 rat brain homogenate; final volume, 3.1 ml. Glucose oxidation: glucose, 0.028 *M*; hexose diphosphate.Mg, 0.005 *M*; succinic acid, 0.0016 *M*; adenosine triphosphate, 0.0007 *M*; DPN, 0.001 *M*; nicotinamide, 0.04 *M*;  $\text{K}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  buffer, pH 7.4, 0.04 *M*; cytochrome *c*, 0.00006 *M*; 0.3 ml. of 1:4 rat brain homogenate from side arm; final volume, 1.1 ml. Anaerobic glycolysis of glucose: glucose, 0.028 *M*; hexose diphosphate.Mg, 0.0025 *M*; adenosine triphosphate, 0.0007 *M*; DPN, 0.0005 *M*; nicotinamide, 0.04 *M*;  $\text{K}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  buffer, pH 7.4, 0.01 *M*;  $\text{NaHCO}_3$ , 0.048 *M*; 0.3 ml. of 1:4 rat brain homogenate from side arm; total volume, 1.0 ml.

Substrate	Drug conc., <i>M</i>	Control	<i>d</i> -Methadone		<i>d</i> -Methadone		<i>l</i> -Methadone	
			cu. mm. $\text{O}_2$	% diff.	cu. mm. $\text{O}_2$	% diff.	cu. mm. $\text{O}_2$	% diff.
Glucose oxidation	0.005	163	57	-65	81	-50	72	-56
Succinate oxidation	0.007	325	87	-74	112	-66	79	-76
Ascorbate oxidation	0.005	381	180	-53	180	-53	95	-76
Glucose, anaerobic glycolysis*	0.005	186	533	+175	635	+241	487	+162
			<i>d</i> -Isomethadone		<i>d</i> -Isomethadone		<i>l</i> -Isomethadone	
			cu. mm. $\text{O}_2$	% diff.	cu. mm. $\text{O}_2$	% diff.	cu. mm. $\text{O}_2$	% diff.
Glucose oxidation	0.005	129	31	-76	58	-55	49	-62
Succinate oxidation	0.007	374	7	-98	7	-98	8	-98
Succinate oxidation	0.003	248	109	-57	106	-57	101	-60
Succinate oxidation	0.003	277	92	-67	113	-59	99	-64
Ascorbate oxidation	0.005	358	60	-84	63	-82	51	-86

\* Values for anaerobic glycolysis are in cu. mm.  $\text{CO}_2$ .

Inhibition of the oxidation of ascorbate, succinate, and glucose by increasing concentrations of *d*-methadone is shown in Fig. 2. The inhibition is approximately the same for all the substrates. Table I shows the comparative inhibition of the oxidation of glucose, succinate, and ascorbate by the methadone isomers. There is no significant difference

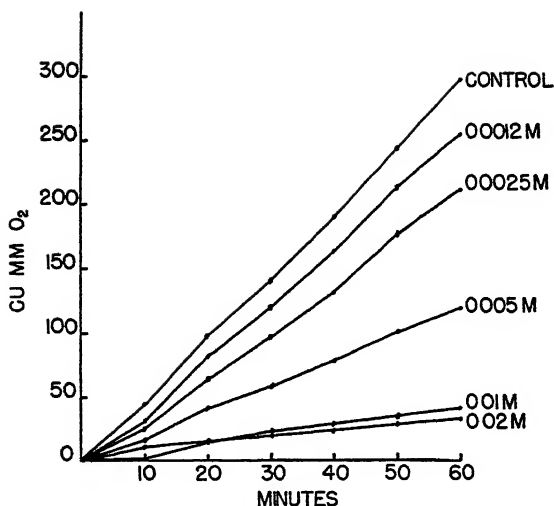


FIG. 1. Inhibition of ascorbate oxidation by increasing concentrations of *dl*-methadone. Each point is an average for duplicate determinations. Experimental conditions same as those given for ascorbate oxidation in Table I.

in the inhibition by the *d*- and *l*- isomers. However, the inhibition by the isomethadone is slightly greater than that by methadone.

Figure 3 and Table I show the effect of the racemic mixture and optical isomers of methadone on anaerobic glycolysis of glucose. Lactic acid determinations on reaction mixtures at the end of 3 experi-

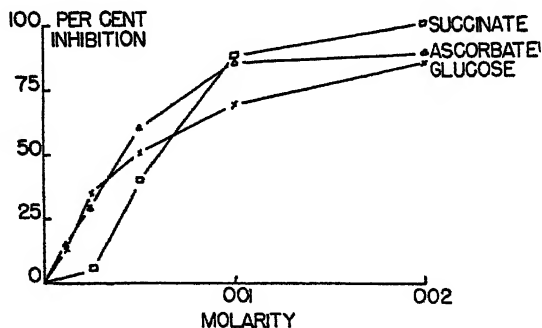


FIG. 2. Effect of increasing concentrations of *dl*-methadone on the respiration of brain homogenates. Each point is an average for 2 determinations. Experimental conditions same as those given in Table I.

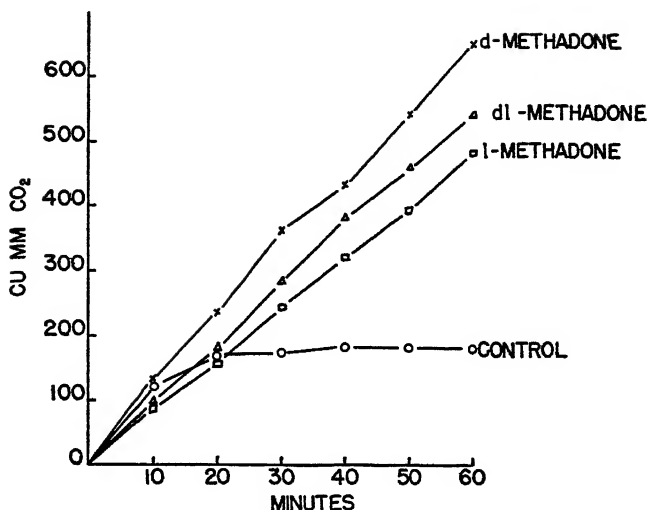


FIG. 3. The effect of methadone derivatives on anaerobic glycolysis. Each point is an average for 3 determinations. Experimental conditions same as those given in Table I. Drug concentration 0.005 *M*.

ments showed the increased carbon dioxide liberation in the presence of the drug was due to lactic acid production.

### DISCUSSION

The *d*- and *l*- isomers of methadone and isomethadone were equally effective as inhibitors of the oxidation of ascorbate, succinate, and glucose by brain homogenates. This is in sharp contrast to the pharmacological and clinical evaluations which have shown that *in vivo* the *l*-isomer is many times more potent than the *d*- form as an analgesic agent.

The methadone derivatives inhibited the oxidation of glucose but did not inhibit the anaerobic glycolysis of glucose at equal concentrations. This indicates the inhibition occurs after the separation of the anaerobic and aerobic phases of glucose metabolism. The inhibition of the oxidation of succinate and ascorbate further indicates the point of blockage is in the oxidative enzyme chain. Since cytochrome-cytochrome oxidase is the only common pathway for the oxidation of all these substrates, inhibition must occur at this level. In a single experi-

ment the rate of oxidation of reduced cytochrome *c* by brain homogenate was followed spectrophotometrically using the method of Albaum, Tepperman, and Bodansky (14). Oxidation of cytochrome *c* was inhibited 85% by 0.005 *M* *dl*-methadone. By adding 0.001 *M* sodium cyanide to the reaction mixture to block the cytochrome oxidase, the reduction of the oxidized cytochrome *c* by succinate was observed to be inhibited 55% by 0.005 *M* *dl*-methadone. These inhibitions were apparently due to enzymatic blockage rather than any direct action of the methadone on cytochrome *c* since increasing the concentration of cytochrome *c* from 0.00007 *M* to 0.0010 *M* had no effect on the inhibition by methadone of the oxidation of succinate.

The increase in carbon dioxide liberated during anaerobic glycolysis in the presence of 0.005 *M* methadone is at least partially due to lactate formation since the amount of lactic acid formed also increases. However, when carbon dioxide production is correlated with time (Fig. 3), activity in the control vessel and vessels with drug is approximately the same for the first 10 min. Then activity in the control vessels rapidly diminishes, whereas, in the presence of methadone, activity continues at the original rate. The increased glycolysis over the 60-min. period is not due to an acceleration but to a maintenance of the original rate of glycolysis. This protection of glycolysis appeared to be of the same type obtained when the destruction of diphosphopyridine nucleotide by diphosphopyridine nucleotidase is prevented by the addition of nicotinamide to the tissue homogenates (10). Several experiments were conducted in which the amounts of diphosphopyridine nucleotide (DPN), nicotinamide, and methadone added to the homogenate were varied in an attempt to show that methadone was inhibiting diphosphopyridine nucleotidase or some other autolytic enzyme. Such a protection by methadone could not be demonstrated.

#### ACKNOWLEDGMENTS

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#### SUMMARY

The effect of methadone derivatives on the oxidation of ascorbate, succinate, and glucose and on anaerobic glycolysis of glucose by rat

brain homogenates was determined by conventional manometric methods. Methadone inhibited the oxidation of ascorbate, succinate, and glucose. Equal concentrations did not inhibit anaerobic glycolysis. The degree of inhibition by the *d*- and *l*- isomers was equal.

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# Isolation of a Pair of Closely Related Antibiotic Substances Produced by Three Species of Basidiomycetes

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## INTRODUCTION

Culture liquids of *Poria tenuis*, *Poria corticola*, and an unidentified *Basidiomycete* from white cedar ("fungus B-841") have been found to possess antibiotic activity against a number of microorganisms, including *Mycobacterium tuberculosis* (1). The work reported here describes isolation of the active principles. Biological studies are presented elsewhere (1).

The active material can be obtained from the culture liquids by extraction with organic solvents, or by adsorption on charcoal. When dried, or allowed to stand in concentrated aqueous solution, it is converted to a dark, brittle lacquer containing only traces of activity. This occurs even when aqueous solutions are freeze-dried.

Two active substances can be separated, which have almost identical bacterial spectra, although they differ markedly in antifungal action (1). They are sharply distinguished chemically by their behavior in solution above pH 7. Substance 1<sup>1</sup> is affected relatively slowly; the change is evidenced spectrophotometrically by gradual decline of the maxima, and biologically by loss of antibiotic activity.<sup>2</sup> Substance 2<sup>1</sup> is affected rapidly, and the change is accompanied by the development of new maxima in the ultraviolet, the most striking of these being an extremely high peak at 242 m $\mu$ . The conversion product of Substance 2 is antibiotic, and has a bacterial spectrum distinct from that of the original compound. It can be isolated readily in the form of crystals, which decompose rapidly on exposure to air.

<sup>1</sup> Substance 1 and Substance 2 are referred to as "nemotinic acid" and "nemotin," respectively, in the biological paper (1).

<sup>2</sup> See Ref. 2, footnote 1, for usage of the terms "antibiotic activity" and "dilution units."

Although the two active substances originally present in the culture liquids have similar ultraviolet absorption spectra, the difference in their behavior toward alkali permitted development of a spectrophotometric method, by means of which the proportion of each compound in a mixture of the two could be determined graphically. Samples of each compound were checked for purity by this method, before being used for further chemical work, or for biological testing.

With the exception of the conversion product of Substance 2, no crystalline derivatives could be obtained.

Elementary analysis of the dried lacquer is negative for nitrogen, sulfur, and the halogens.

Both active compounds show high specific rotations.

On hydrogenation, about 3 g.-moles of hydrogen are taken up per 100 g. of material. The products are colorless waxy solids, which have no antibiotic activity,<sup>2</sup> and no selective absorption in the ultraviolet.

### EXPERIMENTAL<sup>3</sup>

The work described below applies to culture liquids of fungus B-841 except where otherwise indicated. Culture liquids of the other fungi yield the same products, but contain other impurities, and show quantitative differences.

#### *Extraction of Active Material with Organic Solvents*

The instability of the active material made it important to use a solvent which could be readily removed in the presence of water, so that the dissolved material might be transferred directly to aqueous solution, without drying. Ethyl acetate proved to be most convenient. It extracts the activity practically quantitatively, and can be readily removed. Extraction is less efficient with chloroform, and very poor with benzene. Ether is inconvenient for large batches. Methyl isobutyl ketone is efficient as an extractor, but cannot readily be removed.

#### *Extraction with Ethyl Acetate*

Batches of culture liquid of 10–20 l. in volume, varying in activity from 64 to 256 dilution units/ml. were mechanically stirred with ethyl acetate for three 45-min. periods. No adjustment of the pH, which ranged from 4 to 5, was made. The volume of ethyl acetate was 20% that of the culture liquid for the first extraction, and 10% for the second two. The layers were separated in a De Laval centrifuge. The combined extracts were concentrated under reduced pressure to about 5% of their original

<sup>3</sup>The active substances described caused dermatitis in two of the authors (J. P. and F. K.).

volume, and the rest of the ethyl acetate removed under reduced pressure in the presence of a volume of water equal to 1-2% of that of the original culture fluid. The aqueous solution was centrifuged (if not clear), and extracted with 5 times its volume of ether, in 5 equal portions. The substances can be stored in dilute (ca. 0.5%) ethereal solution over sodium sulfate, for periods of at least several weeks, without appreciable change in antibiotic activity<sup>4</sup> or in ultraviolet absorption spectrum.

The data in Table I, which presents averaged values of 15 separate batches of culture liquid of fungus B-841, show that recovery of activity is roughly quantitative. (The term "aqueous solution" is used to indicate the solution obtained by transfer of material from the ethyl acetate extract.)

TABLE I  
*Recovery of Active Material from Culture Liquids*

Size of batch (l.).....	12.6
Activity of culture liquid (dilution units/l.)...	117,200
Activity in aqueous solution corresponding to 1 l. of culture liquid (dilution units).....	108,200
Activity left in culture liquid (dilution units/l.)..	3,700

#### *Adsorption of Active Material on Charcoal*

This method was more rapid, and more convenient for large batches, but its use involved somewhat greater losses of activity.

The culture liquid was stirred with Norit A (Pfanstiehl) (5 g./l.) for 1 hr., and allowed to stand overnight. The charcoal was filtered off, and washed twice with water (2 ml./g. of dry charcoal). The active material was eluted from the washed charcoal by stirring this for 2 half-hour periods with 80% acetone (5 ml./g. of dry charcoal). The acetone was removed under reduced pressure, leaving an aqueous solution. The activity in this solution accounted for about 70% of that in the original culture liquid. The material was transferred to ether, as described above, or was fractionated directly, as described under "benzene fractionation."

#### *Fractionation of Active Material*

Either of two methods was found to be effective: distribution between phosphate buffer (pH 6) and ether, or distribution between benzene and water. The first method gave more consistent results.

#### *Fractionation Using Phosphate Buffer-Ether*

By this method, about half of Substance 1 is obtained readily, free of Substance 2,<sup>4</sup> but purification of Substance 2 from the remainder of Substance 1, is rather laborious. The following example is typical:

<sup>4</sup> Contamination of Substance 1 with Substance 2 is most readily detected by observing the change in absorption above 280 m $\mu$ , on treatment with alkali, since the latter compound under these conditions develops three new peaks in this region, where neither substance originally shows selective absorption. The reverse, presence

An aqueous solution of 100 ml. volume (containing material transferred from an ethyl acetate extract corresponding to 10 l. of culture liquid) is extracted with five 100-ml. portions of peroxide-free ether. The combined ethereal extracts are concentrated under reduced pressure to about 100 ml., and extracted with 100-ml. portions of 0.05 *M* phosphate buffer of pH 6.2. A modified countercurrent method (5 funnels) is used. For complete removal of Substance 1, about 50 extractions with buffer are necessary.<sup>4</sup> Each is run through the 5 funnels. The first funnel contains the original ether extract, the last four, fresh ether. The first 10 buffer extracts contain about 75% of the total amount of Substance 1, free of Substance 2. These are combined, acidified to a pH of about 3, and extracted with an equal volume of ether in 5 portions. Each ether extract is washed, in turn, with three 100-ml. portions of water. Both fractions can be stored in ethereal solution over sodium sulfate.

Both compounds show positive rotation: .

Substance 1, (0.06–0.2% in water) Av.  $[\alpha]_D^{25} + 326$

(0.3 –0.4% in ether) Av.  $[\alpha]_D^{25} + 329$

Substance 2, (0.05–0.1% in water) Av.  $[\alpha]_D^{25} + 314$

(0.4 –0.5% in ether) Av.  $[\alpha]_D^{25} + 250$

Table II shows the distribution of solids and activity between the two substances (averaged data of 5 batches). The figures for Substance 1 are calculated from an ether extract of the first 10 phosphate buffer extracts.

TABLE II  
*Proportion of Active Material Obtained as Substance 1 and Substance 2*

	Activity per vol. corr. to 1 l. culture liquid [1]	Solids per vol. corr. to 1 l. culture liquid [2]	Potency [1] [2]	Per cent recovery, calcd. on basis of 100% for "aqueous solution"	
				Dry wt.	Activity
	<i>units</i> <sup>a</sup>	<i>mg.</i>	<i>units/mg.</i>		
"Aqueous solution"	115,000	100	1150	—	—
Substance 1	46,900	40	1180	40	41
Substance 2	4,800	4.5	1070	4.5	4.2

The proportion of Substance 2 obtained, varied from 7–14%. Spectrophotometric determination on several batches by the method described below, showed the presence of 5–27% in the original mixture, before fractionation.

Another 20% of active material, all Substance 1, is present in the last 40 phosphate buffer extracts. This can be transferred to ether in the same way as the major portion, but the large volumes of buffer make the procedure rather laborious.

of Substance 1 in Substance 2, is more difficult to determine. The criterion of purity used for this compound was failure to obtain from it, on further extraction of its ethereal solution with phosphate buffer, significant amounts of material with the characteristic spectrum of Substance 1, which is unchanged on short treatment with alkali.

### *Fractionation Using Benzene*

This method gives a good yield of both compounds in fairly pure state, but is somewhat inconvenient, due to the formation of emulsions. A countercurrent extraction, using at least 10 funnels, each containing equal volumes of benzene and water, is used. Separation begins with the mixture in aqueous solution. Substance 1 remains in the aqueous phase, Substance 2 is obtained in the benzene.

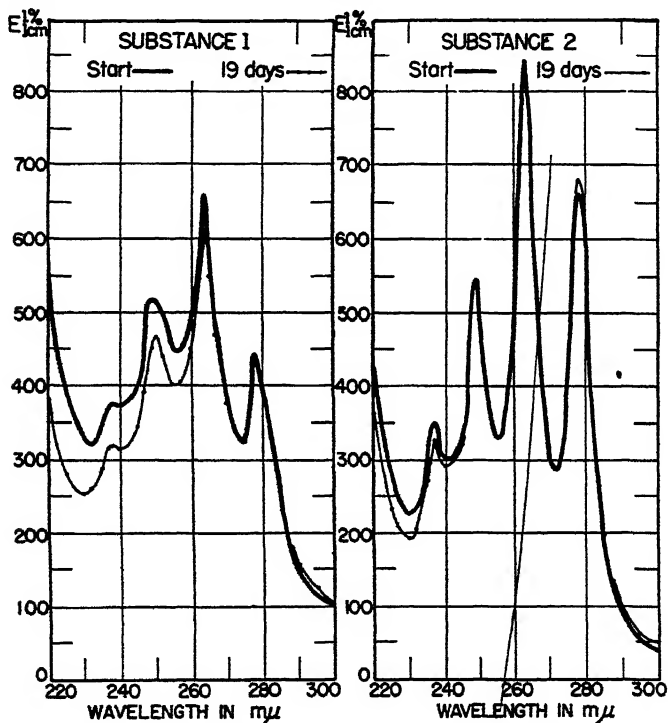


FIG. 1. Ultraviolet absorption spectra of the active compounds, in ether.

### SPECTROPHOTOMETRIC STUDIES

#### *Stability in Ether*

The ultraviolet absorption spectra of the two compounds, after standing in ethereal solution over sodium sulfate in the refrigerator, is shown in Fig. 1. The concentration of Substance 1 was 0.6 mg./ml., and of Substance 2 was 7.4 mg./ml. (In later experiments, the same

results were obtained with much higher concentrations, up to at least 10 mg./ml.). For reading, the solutions were diluted to a suitable concentration with ether. Apparently, there is no significant change in the spectra in the 19 days they were followed.

The antibiotic activity was unchanged.

### *Effect of pH*

For the stability studies at pH 7 and pH 10, phosphate and borate buffers (0.05 *M*), respectively, were used. The material was transferred from ethereal solution to the buffer by removal of the ether under reduced pressure. The relative volumes used were such that the resulting concentration in the buffer was 0.5 mg./ml., the concentration at

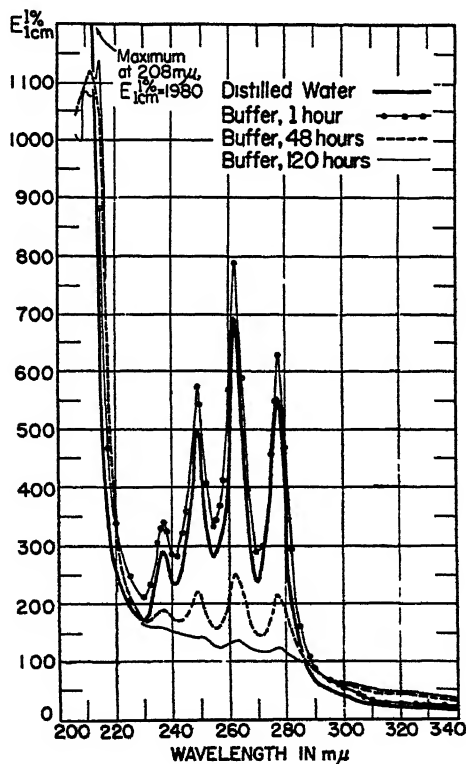


FIG. 2. Change in spectrum of Substance 1 in pH No. 10 buffer at 37°.

which the solution was incubated ( $37^{\circ}$ ). For determination of the absorption spectra, suitable dilutions were made with distilled water. The "blank" cell of the Beckman ultraviolet spectrophotometer was filled with distilled water.

Figure 2 shows the spectrum of Substance 1 in pH 10 borate buffer, after standing various lengths of time. A gradual decrease in the

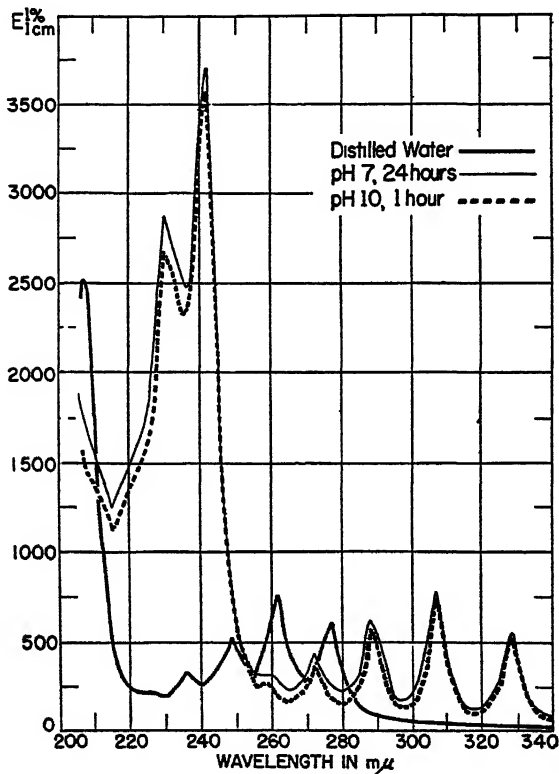


Fig. 3. Change in spectrum of Substance 2 in pH No. 7 and pH No. 10 buffers at  $37^{\circ}$ .

heights of the maxima is apparent, resulting finally in almost complete disappearance of specific absorption. The spectrum in distilled water is shown, for comparison.

The 120-hr. sample had less than 5% of the antibiotic activity of the original material.

In pH 7 phosphate buffer, under the same conditions the spectrum of Substance 1 showed only negligible change after 6 days. The antibiotic activity was unaffected.

Figure 3 shows the effect on Substance 2, of standing at 37° in borate buffer of pH 10 for 1 hr., or in 0.05 *M* phosphate buffer of pH 7 for 24 hr. The compound thus obtained is apparently relatively stable in solution, since this spectrum remains unchanged, under the same conditions, for at least 22 days at pH 7 or 6 days at pH 10 (the length of time for which it was followed). At pH 7, about 16 hr. is required to reach a curve of the form shown.

#### *Isolation of the Conversion Product of Substance 2*

An ethereal solution containing 50 mg. of Substance 2 was concentrated under reduced pressure, and the dissolved material transferred to 50 ml. of pH 7 phosphate buffer by removal of the rest of the ether in the presence of the buffer. The buffer solution was incubated at 37° for 24 hr., and the conversion product extracted with a total of 150 ml. of ether, in 3 equal portions. The combined ethereal extracts were dried over sodium sulfate, and the ether removed under reduced pressure. The residue crystallized in rosettes of needles. The ultra-violet spectrum showed the typical high peak at 242  $m\mu$ , the ratio  $d$  at 242  $m\mu$  to  $d$  at 262  $m\mu$  being 16. On standing in the refrigerator for a few hours, the crystallisate developed black areas, and after 16 hr., a large part had blackened.

#### *Spectrophotometric Determination of the Fractions in a Mixture*

It can be seen from Figs. 2 and 3, that the extinction coefficient of Substance 2 at 242  $m\mu$  rises sharply after incubation of the material at pH 10 for an hour, or at pH 7 for 24 hr., while the curve for Substance 1 is very little affected. Under the same conditions, the absorption at 262  $m\mu$  is somewhat lowered. The ratio of the extinction coefficients at these two wavelengths, after such treatment, therefore, offers a sensitive method of determination of each compound in the presence of the other. The curve obtained when these ratios are plotted against the per cent of Substance 2 in the mixture, is shown in Fig. 4. The points represent values obtained from artificial mixtures of the purified compounds from the same batch, determined at pH 7 and at pH 10.

Table III shows values found for several batches of culture liquid of "fungus B-841," when the active material was isolated by different methods, and the determination carried out at pH 7 and at pH 10.

The figures marked with an asterisk (\*) were determined on a standard curve made from mixtures of Substances 1 and 2 from a different batch from those not so marked. The accuracy of the method

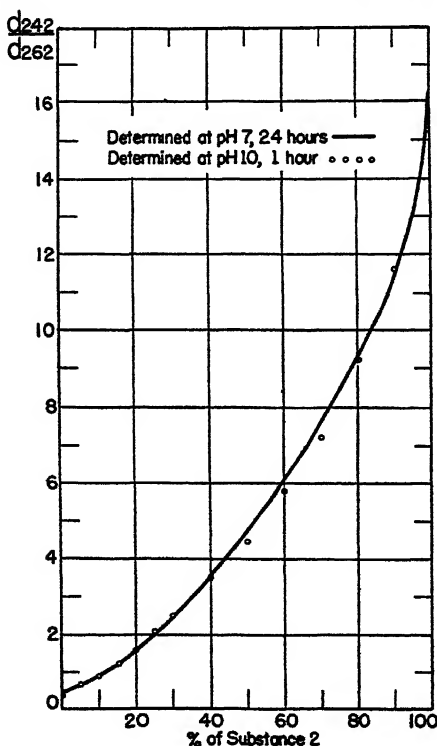


FIG. 4. Standard curves for determining proportions of Substances 1 and 2.

is apparently limited by the duplicability of the standard curve, and can presumably be improved. Ultimately the absolute values will depend on the purity of the compounds used to make the standard curve. Criteria used for determining this are discussed in Footnote 2.

The percentages of Substance 2 in extracts of 3 separate batches of *Poria tenuis* and *Poria corticola* culture liquids were found to be 27, 26, 21, and 29, 13, 28, respectively.

TABLE III  
*Percentages of Substance 2, Determined Spectrophotometrically*

Batch no	Isolation method	pH of incubation	Substance 2 determined	Average for batch
21	Ethyl acetate extraction	10	5, 7**	6
21	Ethyl acetate extraction	7	5, 6*	
22	Ethyl acetate extraction	10	15, 18*	17
22	Ethyl acetate extraction	7	14, 20*	
23	Direct ether extraction of culture liquid	10	10, 11, 12*	12
23	Direct ether extraction of culture liquid	7	10, 14*	
23	Ethyl acetate extraction	10	10, 11, 18*	
23	Ethyl acetate extraction	7	9, 14*	
24	Ethyl acetate extraction	10	25*	25
24	Charcoal adsorption	10	23, 26*	
24	Charcoal adsorption	7	23, 27*	

\* See text for explanation of asterisk.

### HYDROGENATION

Hydrogenation was carried out at room temperature and slightly above atmospheric pressure, in 95% ethanol, using Adams platinum oxide catalyst. (The material was transferred from concentrated ethereal solution to alcoholic solution by removal of the ether under reduced pressure, in the presence of alcohol.) Both fractions consistently took up between 600–750 ml. of hydrogen/g. The products were somewhat waxy white substances, devoid of antibiotic activity.\*

### DISCUSSION

The fact that the two antibiotic substances isolated can be handled only in the form of solutions, makes difficult the accurate quantitative determination of their physical properties. Most of the values reported are averaged from series of determinations, in order to minimize the error. While the spectrophotometric method offers a sensitive check on contamination of the active compounds with each other, the presence of nonabsorbing impurities is not excluded. However, since fairly con-

sistent values are obtained for extinction coefficients (based on dry weight of lacquer obtained from the solution), and for hydrogen uptake for the two substances isolated by several different methods, it seems unlikely that very large amounts of such impurities are present. In the case of Substance 2, the spontaneous crystallization of its conversion product supports this assumption.

The highly unsaturated nature of both compounds is evident from their behavior on drying, and from their ultraviolet absorption spectra, as well as from their high uptake of hydrogen. Similar compounds have been described in the literature: The change in the spectrum of Substance 2 is somewhat similar to that reported by Mowry, Brode and Brown (3) for the alkali isomerization of arachidonic acid: increased absorption below 250  $m\mu$ , and the development of new maxima above 290  $m\mu$ . The change is much more rapid for Substance 2, than for arachidonic acid, and occurs under unusually mild conditions. The similarity in behavior of the conversion product of Substance 2 with the isomerization product of arachidonic acid is striking. The authors state:

"Upon standing in contact with light and air, the material oxidized and polymerized rapidly, becoming sticky, and assuming a yellowish cast. After some time a portion, presumably a polymer, becomes relatively insoluble in hot alcohol."

Kuhn and Grundmann (4) describe decatetraene-2,4,6,8 as being unstable in air, and exploding in oxygen even below its melting point of 125°. The spectrum shows absorption maxima at 272  $m\mu$ , 283  $m\mu$ , 297  $m\mu$ , and 320  $m\mu$ . The maximum at 277  $m\mu$  in Substance 1 and in Substance 2 points to the presence of at least 3 double bonds in conjugation, in these compounds (5-9). The maxima above 300  $m\mu$  in the conversion product of Substance 2 indicate further conjugation in this product (4, 5). It follows that at least 4 double bonds must be present in Substance 2, since it probably can be assumed that none are produced under the mild conditions required for the conversion. Presumably, if a fourth double bond is present in Substance 1, it does not shift to a conjugated tetraene position under these conditions. The sharp difference in behavior of Substances 1 and 2 at pH 7 and above, is particularly interesting in view of the close structural similarity indicated by their original absorption spectra.

Spectrophotometric methods have been used frequently in studies with unsaturated fatty acids (10). Estimation of  $\alpha$  and  $\beta$  eleostearic acids in the presence of one another has been described by Dingwall

and Thompson (6) who used a standard curve prepared by plotting the percentage of  $\beta$  eleostearic acid in mixtures with the  $\alpha$  acid, against  $\log T$  at 2658 Å, a wavelength at which the absorption by these acids showed a large difference. The method presented here is similar to that used by Warburg and Christian for the determination of the enzyme enolase, in the presence of nucleic acid (11), and by Graff and Clarke for the determination of a dye in the presence of hemoglobin (12). The use of a ratio of absorption values at two different wavelengths makes it possible to establish the purity of a sample without determining the total concentration. This is of particular advantage in dealing with unstable compounds of the type described, and is an especially satisfactory method in this case, since the range of ratios is unusually large (about 0.4–15).

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#### SUMMARY

Two closely related, highly unsaturated, and highly unstable antibiotic substances have been isolated from each of three species of *Basidiomycetes*. These substances cannot be handled out of solution since they decompose even on being freeze-dried. An interesting alkali conversion is described, as a result of which a somewhat less unstable compound may be obtained, in crystalline form. The probability that the conversion represents a shift of double bonds to conjugated positions, is discussed. The change apparently occurs with remarkable ease, since similar conversions described in the literature require much more stringent conditions. Procedures for obtaining the active material, and separating it into its two components, are described. A method is presented by means of which the proportion of these components in a mixture, or the purity of either component, can be conveniently determined spectrophotometrically.

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## The Rate of Oxygen Uptake of Differently Prepared Brain Suspensions in the Presence of 2,4-Dinitrophenol <sup>1</sup>

The mechanisms controlling the rates of various metabolic processes in surviving tissues are not clearly understood. One factor determining rates of activity is the method by which excised tissues are prepared for study. Muscle (1) or brain (2, 3, 4, 5) homogenized in water or water extracts of these tissues, show rates of glycolysis many times that found in slices, minces, or Ringer homogenates. Furthermore, water homogenates of brain glycolyze fructose and glucose with equal rapidity, which is not the case with slices or isotonic homogenates (6). Respiratory rate is also known to depend upon the manner in which tissues are prepared (7). Water homogenates of brain show greatly reduced capacity for oxidation when brought back to Ringer glucose phosphate. Although the activity of these preparations can be restored by reinforcing them with various coenzymes and transport-acceptor systems (3) they will not respond to the augmenting effect of dinitrophenol (DNP) (8). In most phenomena involving activity rates there is some tendency to attribute rate differences solely to the concentration of various enzymes (9). The possibility of regulating mechanisms associated with structure has received little attention (2, 6, 8).

We have found that when rat brain is homogenized in distilled water for 5 min. and the concentration is then adjusted to that of Ringer glucose phosphate, or is "reinforced," it responds to  $5.0 \times 10^{-5} M$  DNP only with inhibition of  $O_2$  uptake. Reducing the time of homogenization to 1 min. results in a slight augmentation by DNP. The addition of small amounts of glucose or phosphate buffer has no effect other than that which can be attributed to the change in tonicity of the homogenizing media. On the other hand, homogenates prepared in

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TABLE I  
*Oxygen Uptake*

Rats were killed by decapitation and only the pallium of the brain was used. One ml. of homogenates contained 100 mg. of brain (wet weight). Homogenization carried out in ice-bath. One ml. of homogenate was added to a vessel containing 1.0 ml. of media to bring suspension to that of Ringer glucose phosphate. Equilibration, 15 min. Readings every 30 min. Values given are averages for 1st hr. Controls expressed as cu. mm.  $O_2$ /100 mg. wet weight/hr. DNP expressed as per cent change over control. Gas phase, air.

Type of homogenate	Time of homog.	$O_2$ uptake control	Change, $5 \times 10^{-3}$ M DNP
	<i>min.</i>		%
Water	5	33.4	-41
Water	1	22.7	+23
Glucose <sup>a</sup>	1	21.9	+16
Phosphate <sup>b</sup>	1	44.8	+45
Ringer <sup>c</sup>	1	88.2	+114
RGP <sup>d</sup>	1-5	101.0	+152

<sup>a</sup> Indicates homogenizing media contained 1.8 mg. glucose/ml.

<sup>b</sup> Homogenizing media contained 0.001 M phosphate buffer.

<sup>c</sup> Homogenizing media contained Ringer but neither glucose nor buffer.

<sup>d</sup> RGP = Homogenizing media contained the complete system.

Ringer glucose phosphate or in isotonic solution that preserve *nuclear structure* respond to various concentrations of DNP as do chopped brains. Microscopic examination of water homogenates that showed only inhibition by DNP revealed only abnormal appearing nuclei, and the results of varying the length of time of homogenization in water, or using media of varying tonicity indicated a relationship between the number of normal appearing nuclei and the response to the augmenting effect of DNP. It seems that normal appearing nuclei may be representative of a state of cell organization necessary for the augmenting response in the presence of DNP. This may be due to the presence of such particulates as mitochondria or other subcellular or nuclear entities. Whatever structure is responsible for this effect, it appears that it is inactivated or destroyed by water homogenization. It is also possible that homogenization in water may act by more seriously disturbing the spatial or temporal relationships of the active components than homogenizing in isotonic media. These questions may be answered by procedures that can disrupt the nuclear membrane in

isotonic solution without otherwise exerting any noxious influence on the various vital structures.

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## Heat and Alkali Stability of Pancreatic Lipase

We have noted that glycerolated homogenates of hog pancreas show a distinct difference in their heat stability with respect to the hydrolysis of esters composed of shorter chain acids and monovalent alcohols on the one hand (methyl butyrate, ethyl butyrate, *n*-butyl propionate, *n*-butyl *n*-butyrate) and that of glycerol esters on the other. When glycerolated homogenates of pancreas are heated for 25 min. at 60° the losses in activity for the above mentioned monovalent esters are 93% for methyl butyrate, 100% for ethyl butyrate, 94% for *n*-butyl propionate and 94% for *n*-butyl *n*-butyrate, whereas the corresponding losses for the hydrolysis of glycerol-monobutyryl-, tributyrin-, tripropionin, and olive oil are 60%, 58%, 18%, and 60%, respectively.

More striking results are obtained if protein fractions, obtained from aqueous pancreas homogenates by alcohol precipitation at low temperatures and subsequent dialysis, are treated to different degrees of alkalinity. The following table gives the activity losses incurred by the enzyme, kept for 30 min. at various hydrogen-ion concentrations, with reference to both types of esters. Losses of activity towards the lower monovalent alcohol esters range from 10% to 84%. On the other hand, among the glycerides, monobutyryl is the only one whose hydrolysis is impaired to any extent; this is on the average well less than half of

the corresponding activity losses suffered by the monocarboxylic esters.

Since some hydrolysis of the enzymatic extracts in course of the incubation was observed, a titrimetric method had to be employed by which both the increase in  $\alpha$ -carboxyl groups from the breakdown of the enzyme proteins as well as the amount of acid liberated from the esters could be estimated. Enzyme and substrate blanks were always run concurrently and later deducted. In this respect the formol titration proved superior in exactness to the alcohol titration. Moreover, of all the acids liberated from the esters, oleic acid is the only one which is insoluble in water. Its titration offers however no difficulties if the substrate (olive oil, ethyl oleate) had been emulsified with gum arabic. The experimental results were found to be reproducible in the limits of a maximal error of 2%.

TABLE I  
*Alkali Inactivation of Purified Pancreas Preparation at Varying pH<sup>a,b</sup>*

Substrate	Per cent decrease in hydrolysis after pretreatment at pH			
	7.0-7.2	8.0-8.2	8.6-8.7	9.7-10.0
<i>n</i> -Butyl formate <sup>c</sup>	10		22	54
Methyl butyrate	12	20	38	75
Ethyl butyrate	10	25		80
<i>n</i> -Butyl propionate <sup>c</sup>	21	34	57	82
Ethyl oleate <sup>c</sup>			67	84
Monobutyrim	0	0	8	30
Tripropionin <sup>c</sup>	0	0	0	0
Tributyrim <sup>c</sup>	0	0	0	0
Olive oil <sup>c</sup>	0	0	0	0

<sup>a</sup> Crude pancreas was blended with twice its weight of distilled water. Alcohol was added to 7% and the mixture was kept for 12 hr. at  $-3^{\circ}$ . It was then centrifuged at  $26,000 \times g$  for 15 min. The pellet was resuspended in water and dialyzed against distilled water.

<sup>b</sup> Enzyme kept at the respective pH for 30 min. at  $23^{\circ}$ , and then incubated with the substrate in 0.2 *M* phosphate buffer (pH 8.1), at  $38^{\circ}$ . Present in 26 ml. of digestion mixture was respectively, 2 millimoles of monovalent ester, 2/3 millimole of tributyrin and tripropionin and 0.58 ml. of olive oil. Values given are obtained from end points of a time curve extended over a 1 hr. period and are corrected by deduction of controls. Hydrolysis was estimated by formol titration of 4-ml. samples with 0.05 *N* NaOH using a microburet for this purpose.

<sup>c</sup> Substrate emulsified with gum arabic.

Our observations suggest that the hydrolysis of these two types of esters is related to enzymes which differ in their heat and alkali stability. Attempts to separate the proteins involved are being now undertaken in this laboratory as well as an extension of this investigation to additional esters.

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### Renewal of the Stable Phosphate Group of Adenosine Di- and Triphosphates (ADP and ATP) in Different Organs

In connection with certain isotopic experiments on phosphate turnover in liver and brain it has been shown that the stable phosphate group in ATP and ADP is renewed at a much higher rate than in muscle.

White rats were injected with 0.1 mc labelled phosphate, intraperitoneally in experiments on liver or muscle and subarachnoidally

TABLE I

*Comparison of the Relative Specific Activity of the Stable Phosphate Group of  
ATP in Different Organs after Injection of  $\text{Na}_2\text{HPO}_4$*

Time after inj.	Ref.	Muscle	Brain		Liver	
		Relative specific activity of stable P group <sup>a</sup>	Number of exp.	Relative specific activity of stable P group <sup>a</sup>	Number of exp.	Relative specific activity of stable P group <sup>a</sup>
hr.						
$\frac{1}{2}$	(8)	0	—	—	—	—
1	—	—	—	—	2	33
$1\frac{1}{2}$	—	—	1	28	2	46
2	(8)	3,4	—	—	—	—
3	—	—	1	43	1	72
6	—	—	1	46	2	104
24	—	10 <sup>b</sup>	3	71	2	128

<sup>a</sup> Specific activity of the labile P groups of ATP = 100.

<sup>b</sup> Mean value of 5 determinations, varying between 8 and 12.

<sup>1</sup> On leave from the Hebrew University of Jerusalem; Fellow, National Cancer Institute.

in those on brain, as described elsewhere (1). The animals are sacrificed by drowning in carbon-dioxide-chilled acetone. ATP and ADP were isolated from liver according to Kerr (2), as modified by Rapoport and Nelson (3), from brain according to Kern (4), and from muscle according to Needham (5). Phosphate (6) content and activity (7) were determined by usual methods, after 10 min. hydrolysis in 1N HCl (after correcting for the minute amount of orthophosphate present) and after combustion with  $\text{H}_2\text{SO}_4$ .

Table I shows that the relative specific activity of the stable phosphate group is of another order of magnitude in liver and brain than in muscle.

This seems to agree with the suggestions of Reis (10) and of Kerr (11), according to whom the renewal of adenylic acid proceeds in a different way in liver and brain (possibly through a rephosphorylating action of alkaline phosphatase or nucleoxidas) as compared to muscle (through deamination).

Further exploration of these findings is in progress.

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#### Interference by Sugars and Amino Acids in the Elson-Morgan Method for Hexosamine

The colorimetric method of Elson and Morgan (1) is widely used for the determination of hexosamine in proteins, carbohydrates, and other biological preparations. In attempting to apply the method to a study

of hexosamine synthesis in *Neurospora* we observed that a positive test is given following the addition of various sugars to autolyzates of the mold. It has been found that this does not represent synthesis of hexosamine, but results from non-enzymatic interaction between monosaccharides and certain amino acids, especially lysine, under the conditions of the Elson-Morgan test to produce a red color which is visually indistinguishable from that given by glucosamine. All of the monosaccharides we have tested (glucose, fructose, galactose, sorbose, fucose, xylose) give a positive Elson-Morgan reaction to a greater or less degree even in the absence of lysine; in every case the color is strongly enhanced when lysine—or, to a less marked extent, glycine or arginine—is present. Ketoses react more strongly than aldoses. Lysine does not give the reaction, nor does sucrose, with or without lysine. Carefully inverted sucrose reacts like a mixture of glucose and fructose, however, indicating that the effect is not due to impurities in the monosaccharides.

The tests were carried out by pipetting solutions of sugars, lysine hydrochloride, or glucosamine hydrochloride into test tubes graduated at 10 ml. and adjusting the volume to 1 ml. with water. One ml. of a solution containing 1 ml. of redistilled acetylacetone in 50 ml. 0.5 *N* sodium carbonate was added, and the tubes were heated in boiling water in a shallow bath for 15 min. After cooling, approximately 6 ml. of 95% ethanol was added to each tube, followed by 1 ml. of Ehrlich's reagent (0.8 g. recrystallized *p*-dimethylaminobenzaldehyde dissolved in 30 ml. ethanol followed by 30 ml. concentrated hydrochloric acid. The volume was made to 10 ml. with ethanol and the color read after 30 min. in a Klett-Summerson photoelectric colorimeter, using the green filter.

We have also found that the amount of color developed in the test is strongly dependent on the conditions of heating with acetylacetone. Two to three times as much color develops from the same quantity of glucosamine when the reaction is carried out in 7 × 130 mm. test tubes as when 15 × 150 mm. tubes are used. Furthermore, lysine enhances the glucosamine color in the larger tubes, but not in the smaller ones. These differences appear to be caused by a higher rate of evaporation from the large tubes, since they disappear when these are covered with small beakers. Oxidation by the air seems to play no part in the tube-size effects, since no difference in readings was obtained in covered tubes which had been flushed with oxygen and nitrogen, respectively, before heating. The effect of tube size, or evaporation, on the color developed by fructose in the test is the opposite of that found with

TABLE I

*Reaction of Sugars and Sugars Plus Lysine in the Elson-Morgan Test for Hexosamine*

Reactants	Tube size	Conditions <sup>a</sup>	Colorimeter reading
Reagent blank	mm. 15×150	C, U	0
	7×130	C, U	0
0.1 mg. glucosamine·HCl	15×150	U	80
0.1 mg. glucosamine·HCl	15×150	C	205
0.1 mg. glucosamine·HCl	7×130	U	196
0.1 mg. glucosamine·HCl+1 mg. lysine·HCl	15×150	U	132
0.1 mg. glucosamine·HCl+1 mg. lysine·HCl	7×130	U	199
1 mg. fructose	15×150	U	33
1 mg. fructose+1 mg. lysine·HCl	15×150	U	69
2.5 mg. fructose	15×150	U	79
2.5 mg. fructose+2 mg. lysine HCl	15×150	U	123
2.5 mg. fructose	15×150	C	52
2.5 mg. fructose+2 mg. lysine·HCl	15×150	C	121
2.5 mg. fructose	7×130	U	23
2.5 mg. fructose+2 mg. lysine·HCl	7×130	U	125
1 mg. glucose	15×150	U	15
1 mg. glucose+1 mg. lysine·HCl	15×150	U	35
2.5 mg. galactose	7×130	U	3
2.5 mg. galactose+2 mg. lysine·HCl	7×130	U	56
2.5 mg. sorbose	15×150	C	42
2.5 mg. sorbose+2 mg. lysine·HCl	15×150	C	178
2.5 mg. fucose	7×130	U	4
2.5 mg. fucose+2 mg. lysine·HCl	7×130	U	68
2.5 mg. xylose	15×150	C	9
2.5 mg. xylose+2 mg. lysine·HCl	15×150	C	62
20 mg. sucrose	15×150	U	0
20 mg. sucrose+2 mg. lysine·HCl	15×150	U	5 <sup>b</sup>
20 mg. invert sugar	15×150	U	175
20 mg. invert sugar+2 mg. lysine·HCl	15×150	U	550

<sup>a</sup> U, uncovered tube; C, covered tube.<sup>b</sup> The slight color given by lysine is not red, but yellow or brown.

glucosamine, *i.e.*, a stronger test is given in large uncovered tubes than in small tubes or in large tubes with covers. On the other hand, these factors have little influence on the color developed in the test by mixtures of fructose and lysine. We have also tested the effect on a sugar-lysine mixture of reducing the concentration of hydrochloric acid in the Ehrlich reagent to 0.5 that recommended by Elson and Morgan; no significant reduction in the color was observed.

Sample data are presented in Table I.

These observations have no direct bearing on the Elson-Morgan method for *N*-acetylglucosamine (2), in which acetylacetone is not used.

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### Activation and Stabilization of Aconitase by Ferrous Ions <sup>2</sup>

Aconitase activity decreases quite rapidly in aqueous extracts of mammalian heart (1, 2).

It has been observed in this laboratory that considerable stabilization of crude aconitase solutions can often be achieved by covering them with mineral oil. These observations indicated the possibility of an autoxidizable factor whose presence in the reduced form is essential for aconitase activity.

Addition of  $\text{Fe}^{++}$  to the crude enzyme solution resulted in a slight activation after 3 days, in contrast to the untreated crude solution which lost 80% of its original activity in this period (Table I).

A solution of aconitase dialyzed overnight at 4°C. *vs.* running distilled water was essentially inactive. Incubation of this solution with

<sup>1</sup> Note added after submission of MS.: We have just seen a paper by E. VASSEUR AND J. IMMERS [*Arkiv Kemi* **1**, 253 (1949)] in which the authors also report interference by galactose plus lysine and other amines in the Elson-Morgan method.

<sup>2</sup> These studies were supported in part by a grant from the U. S. Public Health Service.

TABLE I  
*Stabilization and Activation of Aconitase*

Description	Relative aconitase activity after storage for <sup>a</sup>			
	2 hr.	24 hr.	48 hr.	72 hr.
1. Crude, pH 7.0-7.4	0.78	0.31		0.14
2. Crude <sup>b</sup> + Fe <sup>++</sup>	0.70	0.52		0.83
3. Dialyzed crude	0.06	0.06	0.03	
4. Dialyzed crude <sup>b</sup> + Fe <sup>++</sup>	0.32	0.54	0.50	
5. Dialyzed crude <sup>b</sup> + cysteine	0.18	0.64	0.65	
6. Dialyzed crude <sup>b</sup> + Fe <sup>++</sup> + cysteine	0.33	0.77	0.74	

<sup>a</sup> Absorbency readings of the amount of citric acid formed from *cis*-aconitate in phosphate buffer, pH 7.2, 30 min. incubation, 30°C. Citric acid determined by micro modification of method of Speck *et al.* (4).

<sup>b</sup> Preceding the activity determination, the added component was incubated with the enzyme solution for 2 hr. at 7°C. in the absence of mineral oil.

Fe<sup>++</sup> (0.005 *M*) or neutralized cysteine (0.01 *M*) restored considerable activity, whereas incubation with both Fe<sup>++</sup> and cysteine restored aconitase activity to its original value. These results indicate that aconitase requires a cofactor, probably Fe<sup>++</sup>, and in addition must be in the reduced condition to be enzymatically active. Reactivation of a dialyzed solution by cysteine alone indicates that the cofactor is partially nondialyzable under these conditions.

Aconitase solutions prepared by the addition of Pb<sup>++</sup> and subsequent treatment with phosphate to remove Pb<sup>++</sup> are inactive when either Fe<sup>++</sup> or cysteine is added, but some activity is found when the solution is incubated with both Fe<sup>++</sup> and cysteine prior to the activity determination.

Cations tested as activators include Ca<sup>++</sup>, Mg<sup>++</sup>, Ba<sup>++</sup>, Mn<sup>++</sup>, Fe<sup>+++</sup>, Co<sup>++</sup>, Ni<sup>++</sup>, Cu<sup>+</sup>, and Fe<sup>++</sup>. Only Fe<sup>++</sup> additions have resulted in consistent reactivations. The possibility that Fe<sup>++</sup> acts as a cofactor for aconitase is consistent with the inhibition results of Krebs and Eggleston (3).

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## The Presence of Hydrogen Sulfide in Citrus Juices

In a study of the volatile flavoring constituents of citrus fruits, small quantities of hydrogen sulfide were obtained upon distilling grapefruit juice and orange juice at temperatures around 45°C. and under a vacuum of 40–50 mm. of mercury. The question arose as to whether this gas was naturally present in these juices or whether it was formed as an artifact during the distillation. On passage of nitrogen gas through the freshly reamed juices at room temperature into solutions of lead acetate, precipitates of lead sulfide were obtained, indicating that free hydrogen sulfide was present. Semiquantitative analyses yielded 0.45 mg. of hydrogen sulfide/500 ml. of Marsh Seedless grapefruit juice and 0.80 mg./500 ml. of California Valencia orange juice.

Dr. R. J. McColloch of this laboratory has observed that both California Navel and Valencia orange juices give off hydrogen sulfide when treated with activated carbon. Since Nolte *et al.* (1) have also indicated that the mercapto compounds present in orange juice are relatively unstable, it was logical to consider these compounds as possible sources of the hydrogen sulfide. Certain amino acids are known to be present (2) and free cysteine has recently been isolated from orange juice.<sup>1</sup> The azide test for mercapto groups was positive on freshly reamed Marsh Seedless grapefruit juice and on California Valencia juice. The test was faintly positive on California lemon juice and on Mexican lime juice. Indications are that the amount of hydrogen sulfide present in the juice of the fruit varies with the season and the variety.

### ACKNOWLEDGMENTS

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<sup>1</sup> Private communication from E. F. Jansen of Enzyme Research Division, Bureau of Agricultural and Industrial Chemistry; results will be published later.

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## Book Reviews

**Frontiers in Chemistry, Vol. VI, High Molecular Weight Organic Compounds.** Editors: R. E. BURKE and OLIVER GRUMMITT. Interscience Publishers, Inc., New York, N. Y., 1949. vii + 330 pp. Price \$5.50.

This volume of a series is devoted mainly to the physical chemistry of high polymers and is published under the auspices of Western Reserve University. All the chapters, written by experts in various branches of the subject, are, with the exception of the Chapter on proteins by J. T. Edsall, concerned with non-biological macromolecules.

The chapter by W. P. Hohenstein and H. Mark on emulsion polymerization (polymerization of water-insoluble monomers emulsified in water and in the presence of a catalyst, usually peroxide) may suggest to the biochemist the possibility of similar processes occurring in living cells. The chapter on osmometry and viscosity by W. J. Badgley and H. Mark is concerned mainly with flexible uncharged polymers and is, therefore, of limited applicability to most biological substances. H. L. Fisher's discussion on vulcanization, particularly that due to sulfur cross linkages, should be of general interest. J. T. Edsall's chapter is devoted mainly to the physical chemistry of proteins, particularly those fractionated from blood. P. J. Flory has given a detailed account of the kinetics of condensation polymerization and of polyfunctional compounds which can form networks and gels. The chapter by T. S. Carswell is devoted to plastics formed by the condensation of phenols with aldehydes.

All the chapters are well written and each contains a bibliography of about 100 references. There are no new contributions, however, in this volume. Each chapter is either a summary of earlier reviews by the authors or the same review unchanged. Although the material presented can be found elsewhere, some readers may find it convenient to have these reviews available in one volume.

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**Biochemical Preparations, Vol. I.** Chief Editor, HERBERT E. CARTER, University of Illinois. John Wiley & Sons, Inc., New York, N. Y., and Chapman & Hall, Ltd., London, 1949. xi + 76 pp. Price \$2.50.

The contributors and editors of "*Organic Syntheses*" have provided for many years detailed descriptions of convenient methods for the synthesis or isolation of a large variety of organic compounds. Their painstaking work has been greatly appreciated by students and research workers all over the world. Although a considerable number of substances, especially amino acids, have been treated in various volumes of "*Organic Syntheses*," the need for a similar publication, catering specifically to the needs of the biochemist, has been felt for some time. The appearance of the first volume of "*Biochemical Preparations*" will, therefore, give great pleasure to a large number of

biochemists. The general policy of the Editors of the new publication appears to be similar to that followed with such success by the editors of "Organic Syntheses," *i.e.*, to supply full details of the method, to check each preparation or synthesis in at least one laboratory other than that submitting the method, and to use only relatively cheap starting materials and simple apparatus. It is clear that no sharp borderline exists between organic chemistry and biochemistry and a certain amount of overlapping between "Organic Syntheses" and "*Biochemical Preparations*" will thus be unavoidable. It is to be hoped that close contact between the two publications will be maintained.

Volume I covers a wide variety of substances, and the reviewer feels that the selection is wise and not at all one-sided. A number of preparations of amino acids are given, such as L-alanine, L-serine, L-glutamine, L-Dopa and L-lysine. A resolution of tyrosine is also described. It is likely that the recent preparation of D-tyrosine by inversion, described by R. Pitt Rivers and J. Lerman [*J. Endocrinol.* 5, 223 (1948)] is more convenient than resolution. A detailed description of the crystallization of lysozyme is worthy of note. Among other items treated are the preparation of adenosine di- and tri-phosphate,  $\alpha$ -glucose-1-phosphate and diphosphopyridine nucleotide. Typically organic techniques are used in the preparation of DL-glyceraldehyde-3-phosphoric acid. Phosphorylation with diphenylchlorophosphonate is likely to be adopted for a variety of purposes, but may sometimes be replaced by dibenzylchlorophosphonate.

The book is well produced and remarkably free from errors, as far as the reviewer noticed. Literature references are extensive, but not complete. It is to be hoped that the editors of this useful publication will be supported by other biochemists in their endeavor to make available reproducible and convenient laboratory methods.

A. NEUBERGER, National Institute for Medical Research,  
London, N. W. 3, England

**Constitution chimique et activité des molécules thérapeutiques.** By PIERRE LABOUE, Docteur ès-Sciences, Pharmacien, Ex-Interne en Pharmacie des Hôpitaux de Lyon. Masson et Cie, 120 Boulevard Saint-Germain, Paris (6<sup>e</sup>), France, 1948. 118 pp. Price: 310 fr.

Compiled specifically for physicians, the elements of organic chemical structure are reviewed in the first part and the relationships of these structures in some therapeutic compounds are detailed and illustrated in the second part. The recitation of different chemical structures and functional groups in the first part is so brief as to appear inadequate as a source for chemical review by the medical man, perhaps even confusing. In the second part, the author has presented a sound and concise picture of the relations of pharmacodynamic activity to the skeletal structures of various organic drugs together with the effects upon activity caused by modifying these structures with various functional groups.

(The first part could well have been foregone for any sound elementary organic chemistry text, and the second part could have been somewhat expanded, for the author's concept is sound and his presentation is precise.)

Part I. Structure of organic compounds. Review of organic skeletal structures, functional groups, complex (mixed) functional groups.

Part II. Relation between chemical structure and activity of therapeutic compounds. Review of the influence of functional substituents upon activity of some aliphatic, aromatic and heterocyclic structures. Illustration of some important structures found in drugs; modifiers of the central nervous system, the autonomic nervous system, nutrition, metabolism; cardiac stimulants, diuretics, specific antibacterial and bacteriostatic drugs, antiseptics and anthelmintics.

As an aid in picturing the up-to-dateness of coverage of important drugs afforded by this book: antibiotics through penicillin, antihistamines through Neoantergan, anesthetics through Pentothal, analgesics through Demerol, antimalarials through Paludrine, vitamins through folic acid, antithyroids through thiouracil.

ROBERT P. PARKER, Bound Brook, N. J.

**Diet in Relation to Reproduction and the Viability of the Young. Part I. Rats and Other Laboratory Animals.** Tech. Communication No. 16, by F. C. RUSSEL, Commonwealth Bureau of Animal Nutrition, Rowett Inst., Bucksburn, Aberdeen, Scotland, 1948. 98 pp. Price 6s.

This comprehensive review devotes most of its space to studies with rats, but covers the meager literature of research with mice, guinea pigs, Syrian hamsters, rabbits and dogs. It is arranged on the basis of nutrient requirements, starting with stock diets and then dealing, in turn, with the proximate principles, inorganic nutrients, and finally vitamins. The review shows the great gaps in knowledge due, in part, to the confusion that ensues with the discovery of each new vitamin, and the need for constant re-evaluation of older data. In the second place, research in this field has been sporadic, with no single research group devoting its attention to the problems year after year. Since the review was prepared before recent advances with vitamin B<sub>12</sub> had been published, parts of it are already antiquated.

The review draws together the modern literature and should be on the shelves of every worker concerned with laboratory animals. The meager space devoted to dogs covers only literature that is well known. The bibliography is extensive but about three-fourths of it covers American literature that is already familiar.

CLIVE M. McCAY, Ithaca, N. Y.

**Trace Elements in Foods.** By R. W. MONIER-WILLIAMS. Wiley and Sons, Inc., New York, N. Y., 1949. viii + 511 pp. Price \$6.00.

The term "trace element" is applied to those elements which are of importance not only to those interested in the nutritional well-being of plants and animals but also to those interested in public health and physiological safety. Consequently, the biochemistry, nutritional significance, and toxicology of some 40 elements found in the human body and in most foods in amounts up to 0.005% are reviewed and assessed. An attempt is made to describe, often in some detail, quantitative methods for the estimation of many of the elements. References are given for those methods not described.

Although the author has outlined an ambitious program and has developed an adequate formula for the treatment of information pertaining to trace elements, he has followed through in only several cases. For instance, 25% of the book is devoted to 3 elements (copper, lead, and zinc); an additional 25% discusses 5 elements (tin,

arsenic, antimony, selenium, and iron); the third quarter covers 6 elements (nickel, cobalt, manganese, iodine, bromine, and fluorine); the remainder is concerned with 15 elements (indicated in chapter headings) and ten others in a miscellaneous group. As the work progresses, enthusiasm seems to dwindle. Several of these latter substances, such as aluminum, cadmium, chromium, bismuth, mercury, and lithium, deserve more attention than they have received.

The author states that "the analytical sections are in the form of a general outline rather than a detailed laboratory guide." Some analytical sections, however, are the dominant portions of their respective chapters, *i.e.*, one-third of the copper, almost one-half of the lead, one-half of the zinc, and three-quarters of the arsenic chapters discuss analytical procedures. It seems that the emphasis on analysis, in general, is misplaced. This kind of treatment is not of interest or of importance to the general reader and has only suggestive value to research workers in the field.

The book is well documented, but very few references are more recent than 1940. In view of the vast literature pertaining to trace elements that has made its appearance during the past 9 years and, in particular, during the past 5 years, this is an unfortunate situation. Readers will find the book of some value as a summary of earlier work but still must depend upon other sources for more recent material. It definitely does not have "a new look."

E. E. LOCKHART, Cambridge, Mass.

**Physikalische Chemie in Medizin und Biologie.** 2nd edit. By Dr. phil. W. BLADERGROEN. Introduction by Prof. Dr. M. ROCH, Director, Medical Clinic of the University of Geneva. Wepf & Co., Basel, 1949. 650 pp. Sw. fr. 45.

This book, by W. Bladergroen, is a very good text presented under a very misleading title. The physician and the student of biology who will both be inclined to buy this book at the face value of its title, will be disappointed if they expect a new version of the book by Hoeber, "Die physikalische Chemie der Zelle und Gewebe," or a book like Schade's or Becholdt's, which were really text books, showing the application of physical chemistry to medical and biological problems.

However, this book is really excellent as an introduction to the various chapters of modern physical chemistry. The greater part of the book—about two-thirds—is devoted to these problems, and even though formulas like those on p. 83 will not be understandable to most physicians even today, it must be admitted that the author was able to bring most of the difficult problems to a level comprehensible to the biologist with appropriate modern education.

Of special interest are the chapters on nucleonics and isotopes. This is a field of physical chemistry the importance of which in medicine is increasing daily, and in these chapters the lack of adequate medical information is particularly noticeable. In this field, the last few years have produced a great deal of experience in biology as well as in medicine. In the chapter on biological oxidations, as well as in the chapters on quinones, the biological problems are not adequately dealt with. All the work previously done on quinones as specific enzymes of biological importance, as well as their carrier function, is not evaluated.

This is equally true in the chapter on surface tension, which deals in a very clear and simple way with the problems of physical chemistry, but disregards the biological and medical aspects of the problem.

Altogether, the reviewer would say that this is a very good and instructive textbook of physical chemistry, the title of which should be "Physical Chemistry for Physicians and Students of Biology."

BRUNO KISCH, New York, N. Y.

**The Chemistry and Technology of Enzymes.** By HENRY TAUBER, U. S. Public Health Service, Staten Island, N. Y. John Wiley and Sons, Inc., New York 16, N. Y., 1949. viii + 550 pp. Price \$7.50.

When H. Tauber's former book, *Enzyme Technology*, was published in 1943, several reviewers suggested that a section on the chemistry of enzymes should be added to the book. Mr. Tauber however replaced his former book by a newly written one. This is divided into two parts. In Part I, the chemistry of each class of enzymes is treated, while Part II covers the industrial production and use of the same enzymes.

Part I (265 pp.) includes 15 chapters. One chapter is devoted to "General Considerations," 5 chapters to hydrolases (esterases, carbohydrases, phosphorylases, etc., nucleases, proteolytic enzymes). In the following 9 chapters, the author describes the oxidizing enzymes (oxidases, dehydrogenases and other oxidases), the decarboxylating enzymes and the mutases and hydrases. Principally, the classification is the usual one, i.e., that already established in other books on this subject (e.g., Sumner-Somers).

In Part II (250 pp.), the author deals with the industrial production and use of enzymes. It begins with 3 chapters on yeast production and utilization, the production of ethyl alcohol by fermentation, and the role of enzymes in brewing. Then follow chapters on enzymes in microorganisms, namely fermentation by molds (citric acid; gluconic acid, etc.), furthermore, the production of antibiotics by molds, etc., and the production of enzymes from bacteria (e.g., *Bacillus subtilis*) and from molds (e.g., *Aspergillaceae*). In a chapter on enzymes for medicine the author treats pancreatin and other proteolytic enzymes, tyrosinase, histaminase, cholesterinase, etc. Amylases and proteases are used in breadmaking and in the production of malt syrups. The chapter, "Enzymes in Dairy Products" concerns the role of enzymes in cheese making (for the coagulation of milk as well as the ripening of cheese by bacteria or molds), and their use in tests for effective pasteurisation of milk, etc. Typical examples of the roles that enzymes play during storage of foods are the sweetening of grain, potatoes and cheese, due to enzymic hydrolysis, together with the pectolysis of fruits, the desirable hydrolysis of meat, etc., the treatment of fruit juices and wine. A short chapter on vitamin-destroying enzymes (lipoxidase working on carotin; thiaminase; ascorbic acid oxidase) is followed by chapters on enzymes in the textile industries (sizing; degumming; finishing; degumming; laundering) and in the manufacture of leather (dehairing; bating). In Chapter XXXI some very short remarks are made on the use of enzymes in the tobacco industry, in the preparation of drugs and on lipases in the fat industry. The last chapter concerns microbiological methods for the estimation of vitamins and amino acids.

In both parts of the book, a very rich field is treated and many results are summarized. In this connection it will be a very valuable source book to scientists as well as to technicians working on enzymic problems. Sorry to say, the various chapters are not of the same value. There are chapters where the reader perceives the special

interest of the author too much, or where the author has obtained special information, whereas other subjects of general importance are handled relatively too curtly. *E.g.*, 2.5 pp. are devoted to "Surgical Catgut and Enzymes" whereas only 1.5 pp. are given to enzymes in tobacco making; the excellent review of Frankenburg on this subject (80 pp. with 355 references!) has not been considered, while the important findings on enzymes in the fermentation of tea (with the fine work of Roberts) and the fermentation of cocoa are not mentioned at all.

In the scientific part, a great deal of the text is devoted to procedures for the preparation as well as the estimation of enzymes and very detailed descriptions are given. This is of great value to a reader of practical experience, but an undergraduate or graduate student, as well as a reader from some other branch of natural science who is seeking information will be embarrassed by the superfluity of details. Therefore, the reviewer would like to suggest that, in a further edition, the author should lay more emphasis on the general basic principles and should relegate the details somewhat to the background. The book will then gain in clarity. In this connection, the headings of the subdivisions should be revised (see, for instance, the chapter "Carbohydrases" where the application of bold face and of capitals seems to be illogical). The "General Considerations" should be enlarged.

As contrasted with other books (*e.g.*, Sumner-Somers), the historical portion of the text comes off somewhat badly. There are many cases in which only the recent results are mentioned in a very broad way and the older ones are neglected. It is easy to understand that the author wishes to be up-to-date, and he surely is, especially in the results of the war effort, but the reviewer believes that students should also learn of the older investigators on whose shoulders the newer ones are standing.

A. R. F. HESSE, Munich

**Die Penicilline, Arzneimittelforschungen, Vol. 4.** By HANS KILLIAN. Editio Cantor G.m.b.H., Freiburg i. Br., Germany, 1948. vi + 398 p.

This book undertakes making available to German medical scientists, in condensed form, the vast amount of information which has accrued from biological, chemical, and clinical research on penicillin in the Anglo-Saxon countries during and after the war. Barred from access to all but a fraction of the original literature, by import restrictions on Western scientific publications (until recently still in force), the author had to lean heavily on whatever reviews and summaries (largely French and Swiss) he was able to procure, and for the rest on abstracts of lectures and personal communications. It is to his credit that, within the limits imposed on him by the scope and quality of his source material, he has succeeded in presenting a fairly complete, and on the whole accurate, account of the most important developments in the field up to about two years ago. Undoubtedly, the volume will serve the purpose of filling existing gaps of knowledge regarding penicillin in the areas still suffering from dearth of American and British scientific journals, and of providing the background for the understanding of contemporaneous publications which are now presumably again reaching the Central European libraries. By the same token, it is only of negligible interest to the American reader, who has at his disposal not only all of the original literature but also a selection of reasonably up-to-date monographs and review articles covering every aspect of the subject. Although the organic chemist and biochemist in

particular would not find anything in this volume to which he did not have access early in 1946, it must be remembered that, for quite a while thereafter, detailed chemical facts were not easy to obtain, even in this country. Considering the difficulties the author had to contend with in his efforts to assimilate and integrate the disjointed fragments of information within his reach, one must also be charitable about the numerous errors and inaccuracies which mar the discussion of the chemical aspects, such as the notion that the first synthesis of a penicillin (in minute yield) was accomplished by British workers.

In the chapters dealing with the microbiological studies and the clinical applications—subjects in which the author evidently feels more at home than in the chemistry—better literature documentation allows him a firmer grasp of the facts and a closer approximation to the present status of knowledge.

O. WINTERSTEINER, New Brunswick, N. J.

**Pre-Medical Physical Chemistry.** By F. A. MATSEN, Associate Professor of Chemistry and Physics, JACK MYERS, Associate Professor of Zoology, and NORMAN HACKERMAN, Associate Professor of Chemistry, University of Texas. The Macmillan Company New York, 1949. viii + 344 pp. Price \$4.75.

This small volume provides a concise and modern review of those chemical principles which have had application in biology and medicine. The exposition is clear and direct, the development of ideas is logical and coherent. There is an excellent compromise between an elementary and not-too-mathematical approach with rigorous and exact statement.

The first chapter, "Mathematical Introduction," is a very short but effective treatment of logarithms, exponential functions, and differential calculus, with some chemical applications; a reminder of the minimum requisite mathematics. The chapters on atomic and molecular structure, including elementary particles, isotopes, periodic table, and valence, are up-to-date. There follows a rather conventional series of topics: gases, liquids, the solid state, kinetics, equilibrium and free energy, one- and two-component systems, colligative properties, strong and weak electrolytes, oxidation-reduction, pH, surface equilibria, molecular orientation, colloids, electrokinetics, membrane phenomena, and biological energy exchange. Although there is no systematic treatment of thermodynamics as such, the concepts of free energy, activity, and the chemical potential are developed and widely used. Included also are the thermodynamics of oxidation-reduction potentials and of phosphate bond energy. The concept of entropy is not mentioned. The treatment of surface phenomena is more extensive than usual. A desirable feature of the book is the frequent illustration of general mathematical statements by detailed calculation of problems. The problems at the end of most chapters are elementary and serve to check the reader's comprehension.

The book is remarkably free from errors, and shows the result of critical re-examination and careful proofreading. The reviewer has noticed only one minor error: on page 322, in discussion of the condition of 50% oxidation of quinhydrone, it is stated that  $\ln \frac{[q]}{[hq]} = 1$ , instead of  $\ln \frac{[q]}{[hq]} = 0$ .

As might be expected in a text which attempts to cover many ideas in a small space, the treatment of some ideas is too brief to be logically convincing, and many statements must be accepted on faith. In other words, when used as a teaching text, this

book leaves considerable for the instructor to amplify, to illustrate, to justify, and to prove. This is true for example of the subjects of liquid junction potentials, the glass electrode, buffers, and the Donnan equilibrium in the blood. Although original literature is not cited, reference is made to a few books at the close of each chapter. Further references especially to review articles would be of value, not only to stimulate the student to inquire further, but to help him realize that our knowledge of the subjects treated is far from being encompassed by this one book.

In a book which is refreshingly modern in its treatment of most topics, it was surprising to find, on page 176, in the mathematical evaluation of the hydrogen-ion concentration of an amino acid from its ionization constants, that the second ionization of the amino acid is depicted as involving the hydration of the molecule, and dissociation of a hydroxyl ion. The use of Brønsted's idea of the formulation of this ionization as an association of a proton by the negatively charged carboxyl group of

the dipolar ion,  $^-\text{OOCRNH}_3^+ + \text{H}^+ \longrightarrow \text{HOOCRNH}_3^+$ , would have greatly simplified the mathematical formulations.

The value of the book for biologists could be improved if it included more extensive treatment of certain important biochemical applications of physical chemistry. For example in connection with the subjects of kinetics and enzymes, the idea of the enzyme-substrate association constant is not mentioned. In connection with biological energy exchange, the stepwise liberation of energy of oxidation, by the successive transfer of electrons to a variety of mediators is not developed. The relation of the Donnan membrane theory to the physicochemical properties of proteins is not discussed. The exposition frequently ends abruptly with a statement of the physicochemical principles, when, to a biochemist, it seems unfortunate, after having laid such a good foundation, not to show where these principles have had application.

The directness of statement and brevity of the book allow one to see many ideas in a short time and make for stimulating reading. The diversity of subjects treated gives a satisfactory coverage of physical chemistry. The book should have value as the text of a course for premedical students, and as a source of information for biologists generally.

MARTIN E. HANKE, Chicago, Illinois

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# Inhibition of Growth of *Lactobacillus casei* by Methionine and Its Relation to Folic Acid Assimilation<sup>1</sup>

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Received May 24, 1949

## INTRODUCTION

Reports on the role of methionine in the nutrition of *Lactobacillus casei* are contradictory. A study of Hutchings and Peterson (2) suggests a limited requirement or stimulatory function of methionine. Dunn and co-workers (3) observed complete growth in a methionine-free medium. According to Evans (4) methionine is required and can be replaced by homocystine and choline. We have been unable (5) to confirm the findings of Evans and at the same time have encountered a strong inhibitory effect of methionine on the growth of *L. casei* in the absence of norleucine (which is no longer considered a naturally occurring amino acid). The present paper deals with a partial investigation of this hitherto undescribed phenomenon.

## EXPERIMENTAL

### *Organisms, Medium, Bacterimetric Technique*

Four different cultures of *L. casei* 7469 were used: one obtained directly from the American Type Culture Collection, and the others from the State College of Washington, Pullman, Wash. They were carried in Difco bacto-yeast dextrose agar (per liter: 5 g. bacto-peptone, 3 g. bacto-beef extract, 2.5 g. bacto-tryptone, 1 g. bacto-yeast extract, 1 g. bacto-dextrose, and 15 g. bacto-agar).<sup>3</sup> Since all cultures showed the same responses the present findings may be considered valid for *L. casei* 7469. For the growth of inocula, the peptone-yeast extract medium of Riesen *et al.* (6) was used.

<sup>1</sup> Aided by a grant from the Mary Frances Sarkisian Memorial Fund. Presented in part at the 1948 meetings of the American Chemical Society, Washington, D. C., and Portland, Ore. (1).

<sup>2</sup> Deceased December 6, 1948.

<sup>3</sup> We are indebted to Miss Marian Setterlund for the cultures and the information.

The medium used in this work is patterned in detail after that actually used (4) by Evans,<sup>1</sup> except that norleucine was omitted.

The concentration of its components, per milliliter of finished medium, is as follows:

- 20 mg. each of glucose and anhydrous sodium acetate,
- 2.5 mg. each of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ ,
- 0.4 mg. each of DL-alanine, DL-glutamic acid and L-asparagine,
- 0.2 mg. each of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , L-arginine, L-cystine, glycine, L-histidine, DL-isoleucine, DL-leucine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, and DL-valine,
- 0.1 mg. of L-lysine,
- 10  $\mu\text{g}$ . each of adenine, guanine, uracil,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , and NaCl,
- 0.5  $\mu\text{g}$ . each of nicotinic acid, pantothenic acid, riboflavin, and thiamine hydrochloride,
- 0.2  $\mu\text{g}$ . of pyridoxamine,
- 0.1  $\mu\text{g}$ . of *p*-aminobenzoic acid,
- 4  $\mu\text{g}$ . of biotin, and
- 2  $\mu\text{g}$ . of folacin.

The bacterimetric procedures have been previously described (7). The spectrophotometric turbidity values (875  $\text{m}\mu$ ) are directly proportional to tube size and can be corrected for deviations from Beer's law by a standardized optical density adjustment formula (8).

### *Inhibition by Methionine*

Fig. 1 shows the growth responses which result from the presence of increasing amounts of DL-methionine. As little as 10.025  $\mu\text{g}$ . of methionine/ml. has a measurable inhibitory effect. The effect increases with increasing concentrations of methionine. The shape of the growth curves suggests that the primary effect of methionine consists in lengthening the lag phase of growth. Once growth has set in, the curves run a nearly parallel course, *i.e.*, methionine causes a delay following which the growth rate tends to become normal. Furthermore, the curve representing 6.4  $\mu\text{g}$ . of methionine indicates acceleration relative to the effect of the next lower concentration.

The data recorded in Table I indicate that the inhibitory activity of methionine is confined to the L-stereoisomer.

### *Dependence of Methionine Effect on Stage of Bacterial Growth*

The generally parallel character of the growth curves suggests that the inhibition is no longer effective when the lag phase has been passed. In order to clarify the function of methionine after logarithmic growth has begun, and the significance of the positive response shown by the 6.4  $\mu\text{g}$ . level, an experiment was designed in which a series of tubes, initially without methionine, were supplemented with graded quantities of methionine in a sterile solution, after 24 hr. of growth. The results of this experiment are shown in Fig. 2. It is evident that when added at this stage, methionine not only fails to inhibit but definitely accelerates further growth.

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<sup>1</sup> We are indebted to Dr. Evans for the details.

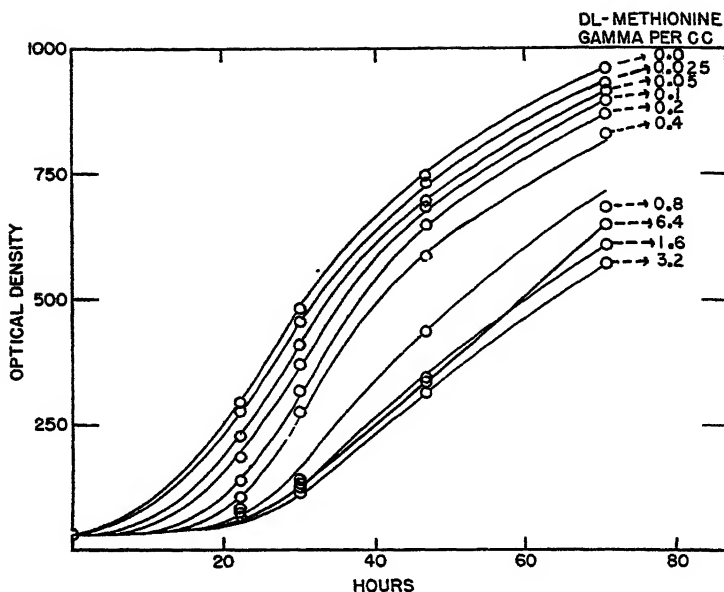
FIG. 1. Effect of methionine on *L. casei* (tubes 13.4 mm. i. d.).

TABLE I  
*Comparison of DL-, L-, and D-Methionine*

Supplement  <i>μg /ml.</i>	Optical density (15.8 mm. tubes)			
	24 hr.	31 hr.	48 hr.	54 hr.
None	405	595	925	1000
0.33 DL-Methionine	180	390	810	890
0.67 DL-Methionine	110	250	670	765
1.04 DL-Methionine	105	150	535	625
0.17 L-Methionine	165	370	770	865
0.33 L-Methionine	110	240	665	755
1.00 D-Methionine	140	330	725	820

L-Methionine:  $[\alpha]_D^{25} = +21.4^\circ$  (0.8% in 0.2 N HCl), i.e.,  $98 \pm 1\%$  L-compound [cf. (6)].

D-Methionine\*:  $[\alpha]_D^{25} = -12^\circ$  (1.0% in 0.2 N HCl), i.e.,  $23 \pm 2\%$  L-compound [cf. (6)].

\* We are indebted to Dr. J. A. Stekol for this product, obtained by him by resolution of synthetic DL-methionine.

It seemed desirable to consider next whether the changing response to methionine is related to an adaptation of the organism to the medium or to some change in the composition of the medium which accompanies the termination of the lag phase. For this purpose a quantity of the described medium was distributed in tubes and sterilized as usual. One group of tubes (*a*) was stored in the cold and the others (*b*) were inoculated and incubated for 17 hr. At this time the bacterial density corresponded to approximately OD 300 (in 15.8 mm. tubes). The contents of the (*b*) tubes were combined, centrifuged, filtered through paper and then through a sterile UF Pyrex glass filter. The filtrate was distributed into sterile tubes (*b'*).

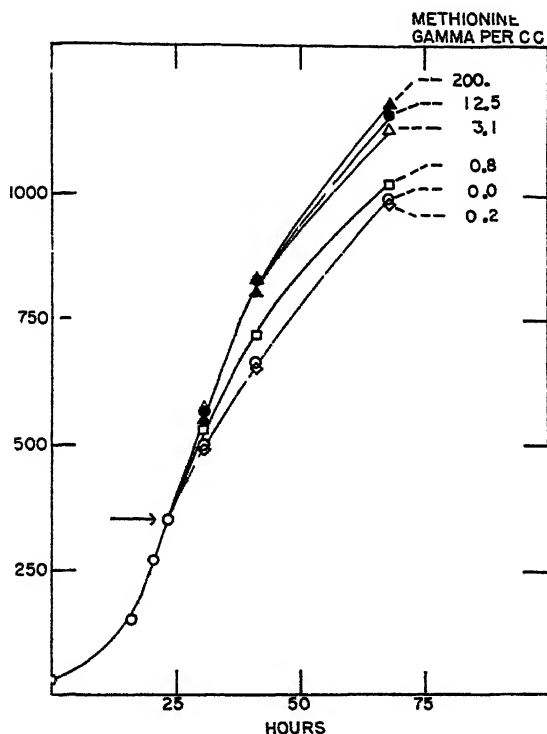


FIG. 2. Effect of methionine during the logarithmic phase of growth. Methionine was added at the point indicated by the arrow (tubes 13.4 mm. i. d.).

Within each of the two groups (*a* and *b'*), pairs of tubes were now sterilely supplemented with methionine in quantities corresponding to 0, 0.05, 0.2, and 1.0  $\mu\text{g.}/\text{ml.}$  All tubes, with the exception of sterility controls for each group, were inoculated and all tubes were incubated. After 24 hr. the (*a*) group showed growth in all tubes, with the expected graded decrease in bacterial density ranging from 450 in the methionine-free tubes to 100 in the tubes containing 1  $\mu\text{g.}$  of methionine/ml.

There was virtually no growth in any of the (b') tubes. This was wholly unexpected since the initial medium is "complete", *i.e.*, it presumably contains all nutrients in quantities well above their growth-limiting levels: in the unfiltered (a) tubes density levels of 1200 to 1300 are obtained after 3 days of growth. Since in the filtered tubes (b'), growth prior to filtration [(b) tubes] had been allowed to reach only the 300 level, substantial growth after reincubation was expected.

### *Role of Folacin in Methionine Effect*

Systematic experiments revealed that only the addition of folacin is required to re-establish the growth potential of the filtered tubes. Since without filtration growth proceeds to high density levels it appears that in the early stages of proliferation the cells accumulate a large excess—in the present case all that is available—of folacin and thus deplete the medium. Presumably the accumulated folacin is divided among the progeny during subsequent cell divisions until the internal concentration drops to a growth-limiting level.

The experiment just described was repeated with the modification that the medium in which bacteria had been grown to a density of 300 was, after filtration, resupplemented with the original quota of folacin. Increasing inhibition of growth with increasing amounts of methionine in this medium after reincubation showed that a change in the composition of the medium—such as the appearance of an exogenous metabolic factor in the medium during early growth—is not what is responsible for the reversal of the methionine effect which is apparent when methionine is added after logarithmic growth has begun (see Fig. 2).

The possibility now appears that bacterial hoarding of folacin and growth inhibition by methionine are related phenomena since both occur during the early phases of cell multiplication. In the next experiment a series of tubes were again allowed to grow to a density of 300. At this point additions were made to pairs of tubes of: (a) nothing, (b) 1  $\mu\text{g.}/\text{ml.}$  of methionine, (c) 0.002  $\mu\text{g.}/\text{ml.}$  of folacin, and (d) both methionine and folacin in the amounts used in (b) and (c). The results are shown in Fig. 3. As expected, methionine accelerates slightly at this stage. However, the addition of further folacin causes substantial stimulation and this stimulation is clearly counteracted by methionine. The preceding evidence had indicated that at the 300 stage of growth the medium has become essentially folacin-free. Addition of folacin now results in further assimilation of this metabolite by the increased bacterial population, accompanied by an accelerated growth rate, and the evidence suggests that the inhibitory effect of methionine is exerted via interference with folacin assimilation.

This theory, assuming interference of methionine with folacin assimilation, was subjected to further tests. If the theory is correct the stage of bacterial proliferation at which methionine ceases to be inhibitory should coincide with the stage at which folacin accumulation from the medium has gone to completion. To test this, a series of tubes containing the methionine-free medium was inoculated and incubated. At different periods, beginning 10 hr. after inoculation, pairs of tubes were sterilely supplemented with 1  $\mu\text{g.}/\text{ml.}$  of methionine and returned to the incubator. At the same periods other pairs of tubes were filtered through UF filters, and the filtrates were

stored in the refrigerator. Finally, all filtered tubes were resterilized, inoculated and incubated. After 47 hr. of incubation one tube of each pair of the filtered tubes was sterilely supplemented with  $0.002 \mu\text{g.}$  of folacin and returned to the incubator. Figs. 4a and 4b illustrate the nature of the growth responses observed in this experiment. Fig. 4a shows, in confirmation of previous observations, that the inhibitory effect of added methionine tends to vanish after 18 hr. of incubation as growth approaches the 300 level of optical density. Similarly, Fig. 4b suggests that depletion of the medium of folacin is nearly complete after 18 hr. It is apparent that after approximately 12 hr. of growth both the effective level of folacin and the inhibitory effectiveness of methionine have been reduced by one-half. Thus, the observed relations are broadly consistent with the theory.

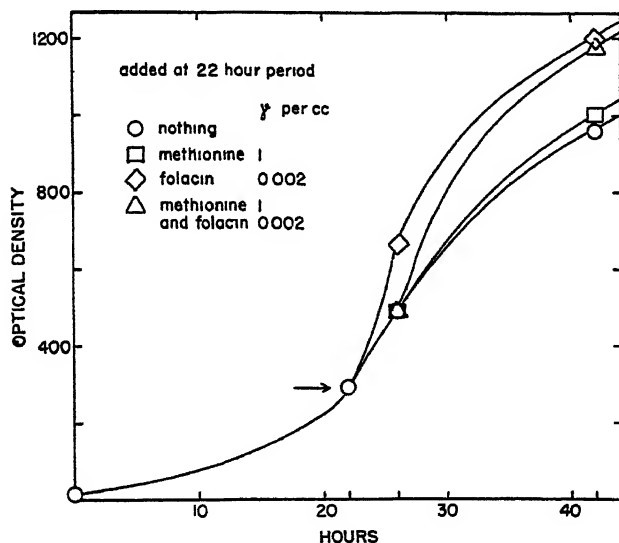


FIG. 3. Effect of methionine on growth stimulation by folacin (tubes 13.4 mm. i. d.).

The possibility was now considered that the apparent relation between methionine and folacin may be competitive in nature. The results of an experiment in which both the folacin content and the methionine content of the medium were varied over a wide range are shown in Fig. 5. It is evident from this experiment that the folacin concentration required for the attainment of the maximum growth rate in the present medium is approximately 10 times as large as that used in the preceding experiments ( $0.002 \mu\text{g./ml.}$ ). This finding was foreshadowed by the results shown in Fig. 3, where folacin supplementation beyond the initial level resulted in enhanced growth. The experiment further shows that the inhibitory effect of methionine remains essentially

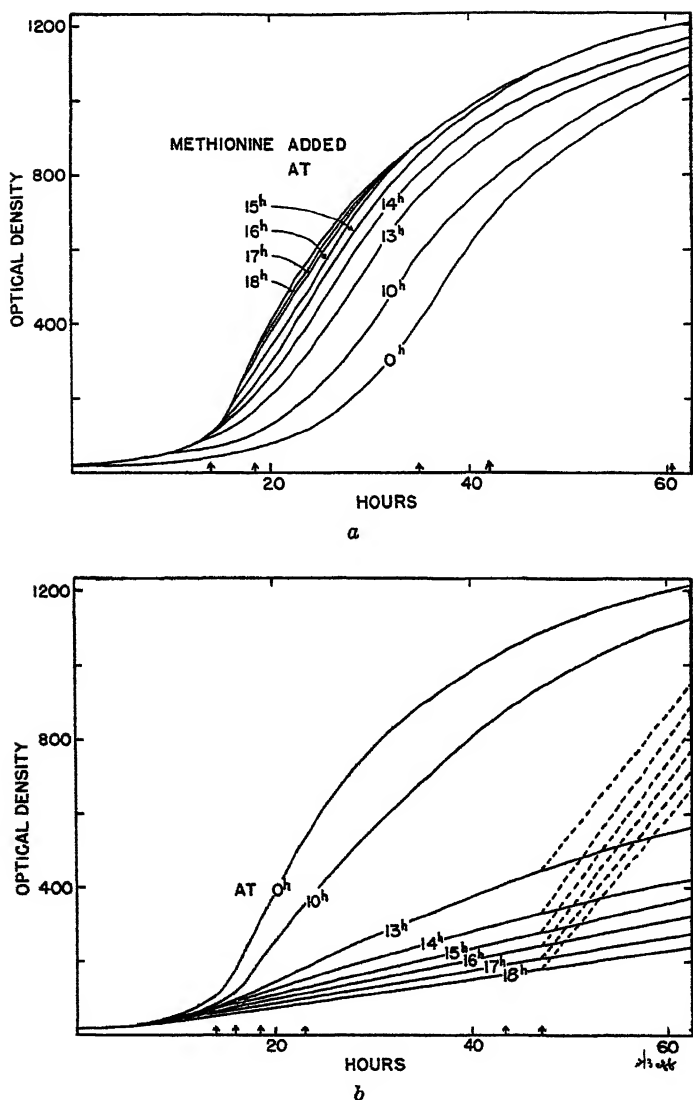


FIG. 4. Relation between inhibitory action of methionine and folacin depletion of medium (tubes 15.8 mm. i. d.). Arrows on abscissa indicate time of measurements. Upper chart (a): Effect of methionine addition (1  $\mu\text{g./ml.}$ ) at different times. Lower chart (b): Effect of filtration at different times upon growth after reinoculation. The dotted lines show the response produced by the addition of folacin (0.002  $\mu\text{g./ml.}$ ) to

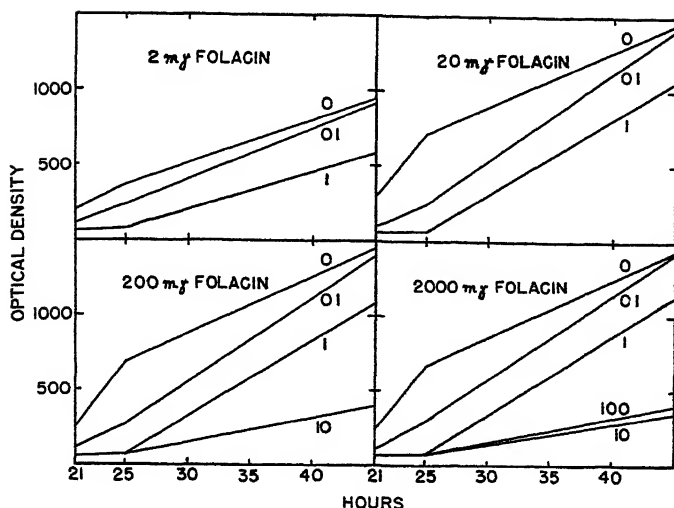


FIG. 5. Effect of methionine in the presence of different concentrations of folacin (tubes 15.8 mm. i. d.). The figures on the curves indicate  $\mu\text{g. DL-methionine/ml.}$

the same when the folacin concentration is increased one-hundred-fold beyond the level required for optimal growth, so that the presumed effect of methionine on folacin assimilation appears to be of a noncompetitive nature.

### *Development of a Methionine-Resistant Strain*

When organisms grown in the presence of methionine in the synthetic medium were used as an inoculum for this medium, a reduced inhibitory effect of methionine was

TABLE II  
*Development of Methionine-Resistant L. casei*

DL-Methionine $\mu\text{g./ml.}$	Optical density (15.8 mm. tubes)								
	Ordinary inoculum	Inoculum A		Inoculum B		Inoculum C		Inoculum D	
	41 hr.	25 hr.	71 hr.	25 hr.	71 hr.	19 hr.	25 hr.	19 hr.	25 hr.
0	990 (A)	570	1155 (C)	475	1185	110	440	215	480
0.05	915	430	1135	430	1165	60	295	220	480
0.2	840	275	1090	395	1155	40	135	250	525
1.0	525	95	955	400	1180 (D)	45	75	285	545
2.0	480 (B)								

A capital letter in parentheses indicates that the culture was used for the preparation of the inoculum identified by the same letter.

encountered. The data of Table II show that in a culture grown once in the presence of methionine (inoculum *B*), the negative response to methionine still predominates. However, after a second transfer from a methionine-containing medium (inoculum *D*) an actively growing strain has resulted whose response to methionine is completely reversed.

### DISCUSSION

The three main observations encountered in this study are (a) the capacity of L-methionine to lengthen the lag phase of *Lactobacillus casei*, (b) the capacity of the organism to accumulate folacin to the point of virtual depletion of the medium, and in quantities which cover the metabolic requirements of several future generations of cells, and (c) the apparent interference of methionine with folacin assimilation as the primary cause of the inhibitory effect of methionine during early growth.

The literature contains observations which are related to the finding noted under (b). Gale (10) has shown that bacterial cells accumulate free amino acids in quantities which are of the order of 50% of those contained in the cellular protein and that at "saturation" the internal amino acid concentration may be 5 times as high as the external concentration. Substantial "carry-over" of vitamins in washed inocula is well known. Specifically, Krueger and Peterson (11) have shown that in the presence of an excess of the vitamin bacterial cells can accumulate 10-15 times the amount of biotin required for maximum growth. The present data indicate that vitamin hoarding may take place even in the absence of an excess; in fact, the data suggest that the cells will not utilize their vitamin hoard for the production of additional cells, *i.e.*, growth, at maximum rate until their hoarding capacity has been saturated. The experiment illustrated in Fig. 4 indicates that the rate of assimilation of folacin is so much greater than the rate of its utilization for cell multiplication that after 13 hr. of growth enough of the vitamin has already been hoarded with three-quarter depletion of the medium<sup>6</sup> to permit an approximately 30-fold growth in population, *i.e.*, the formation of four to five additional generations. While the present data permit no decision as to whether folacin is stored free or in a conjugated form, the evidence does indicate that substantially all of it is available and in that sense equivalent to folacin added in the medium.

<sup>6</sup> Data presented elsewhere (8) indicate that an OD of 1300 corresponds to a relative bacterial concentration of approximately 3000.

Since methionine has been shown (12) to be a tissue component of *L. casei* (0.5% of its dry weight) and since our experiments, as well as others (2), show that it is stimulatory under certain conditions, it must be classed as a—potential if not obligatory—nutrient for the organism. Accordingly, its inhibitory effect, if it involves the utilization of another nutrient, folacin, appears as a case of nutrient antagonism. Examples of nutrient antagonism are known in bacterial nutrition, such as for instance the antagonisms between tryptophan and phenylalanine (13), threonine and serine (14), or pyrimidines and serine (15). The present case seems unique in that it involves an amino acid and a vitamin, in that it does not appear as a reversibly competitive process, and in that the concentrations of the amino acid which are inhibitory are of a definitely lower order of magnitude than those which are nutritionally effective.

The rapid emergence of a methionine-resistant variant (Table II) complicates precise interpretation. The nutritional requirements of the resistant strain, as well as the possibility of its reversal to the sensitive strain, call for investigation. As noted, the natural strain was grown on inoculum media containing peptone, yeast extract, or enzymatic casein hydrolyzate. Thus either the methionine in these media is present in a noneffective peptide linkage, or other factors counteracting the methionine effect are operative.

#### SUMMARY

1. In certain synthetic media characterized by the presence of asparagin and the absence of norleucine, *L*-methionine inhibits early growth of *L. casei* 7469.

2. During early growth the organisms accumulate the folacin requirements of several future generations. After depletion of the medium, growth continues at the expense of the hoarded folacin.

3. After bacterial accumulation of folacin has ceased, methionine stimulates growth.

4. The evidence suggests that as an inhibitor, methionine acts by interfering with the assimilation of folacin, and as a stimulator, by serving as a protein building block, sparing cystine.

5. The postulated relation between folacin and methionine has no reversible characteristics.

6. After several cultures in the methionine-containing synthetic medium, a variant of *L. casei* has developed which responds positively to methionine.

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# Metabolism of C<sup>14</sup>-Labeled Tyrosine and Phenylalanine in Phlorizinized Rats <sup>1</sup>

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## INTRODUCTION

The use of isotopically labeled compounds introduces a new approach to the study of intermediary metabolism using diabetic animals. The oxidative mechanisms for the three major foodstuffs in the organism, leading finally to the formation of carbon dioxide, include oxidative decarboxylation and oxidations that result in the formation of intermediary smaller units. An indication of the mechanism involved and of the nature of the intermediates can be obtained by locating the position of the isotope in the excreted glucose and ketone bodies of diabetic animals. The experiments described in this paper were carried out with this aim. While these experiments were in progress, *in vitro* experiments using labeled tyrosine and phenylalanine have been reported (1, 2) which are confirmed by our data.

Tyrosine and phenylalanine are generally considered to be ketogenic amino acids since they increase ketonuria in phlorizinized rats (3, 4) and produce acetoacetate on perfusion of surviving livers (5) or incubation with liver slices (6). Winnick, Friedberg, and Greenberg (7) observed that radioactive ketone bodies were formed in a normal rat after administration of C<sup>14</sup>-labeled tyrosine. In the present experiments, it has been determined that the beta-carbons of tyrosine and phenylalanine enter ketone bodies and glucose.

<sup>1</sup> Supported by grants from the Committee on Research in Endocrinology, National Research Council, The John and Mary R. Markle Foundation, and the Research Committee of the Medical School.

<sup>2</sup> Government of India Fellow. Present address: Enzyme Institute, University of Wisconsin, Madison.

## EXPERIMENTAL

*Administration of Labeled Compounds*

The DL-tyrosine<sup>3</sup> and DL-phenylalanine<sup>3</sup> used in the study were labeled on the beta-carbon of the side chain with C<sup>14</sup> and had activities of 325,000 counts/min./mg. and 3,030,000 counts/min./mg., respectively. In order to decrease the C<sup>14</sup> incorporation into the proteins and produce appreciable urinary excretion, the amino acids were diluted in the following manner.

Approximately 4 mg. of the isotopic DL-tyrosine was dissolved in 1 N HCl containing 600 mg. of nonisotopic DL-tyrosine, and the mixture precipitated at pH 5.8. This diluted product is referred to as "DL-tyrosine." In the next experiment, 4 mg. of the labeled DL-tyrosine was mixed with 600 mg. of inert L-tyrosine, dissolved completely in boiling water and evaporated to dryness *in vacuo* ("L-tyrosine").

TABLE I  
*Radioactivity of Urinary Components*

Rat	Compound injected <sup>a</sup>	Urine		Glucose			Ketone bodies			Urea counts/ mun./ mg.
		Total counts/ min.	Per cent of dose	Excreted mmoles	Counts/ min./ mmole	Per cent of dose	Excreted mmoles	Counts/ min./ mmole	Per cent of dose	
1	"DL-tyrosine"	9,320	2.43	5.66	564	0.55	1.68	1,530	0.45	82
2	"DL-tyrosine"	8,400	2.33	5.91	398	0.51	1.93	984	0.45	95
3	"L-tyrosine"	82,800	15.4	5.66	4,540	5.1	1.55	19,900	6.10	210
4 <sup>b</sup>	"L-tyrosine"	184,000	23.5	2.76	4,760	2.3	0.413	16,600	1.20	160
5	"DL-phenylalanine"	179,000	30.5	4.56	6,230	4.83	2.08	15,900	5.63	296
6	"DL-phenylalanine"	198,000	33.7	5.1	6,150	5.33	1.82	11,200	3.47	185
7	"L-phenylalanine"	83,800	14.3	5.97	4,250	4.32	4.41	12,900	9.66	207
8	"L-phenylalanine"	81,800	13.9	5.33	4,480	4.07	3.80	12,800	8.34	279

<sup>a</sup> Radioactivity absorbed (counts/min.  $\times 10^{-3}$ ) Rat 1, 384; Rat 2, 360; Rat 3, 506; Rat 4, 574; Rats 5-8, 588.

<sup>b</sup> Rat 4 died 8 hr. after the injection.

The radioactive DL-phenylalanine (0.47 mg.) was diluted with 300 mg. of inactive DL-phenylalanine in one case (referred to as "DL-phenylalanine") and with 300 mg. of inactive L-phenylalanine in the other ("L-phenylalanine").

Rats were phlorizinized as reported in a previous communication (8). On the third day after the onset of phlorizin administration, the diluted tyrosine was injected subcutaneously as an aqueous suspension at two different sites, and the diluted phenylalanine injected in aqueous solution. Urine was collected for 24 hr.

The rats were autopsied at the end of the experiment and it was found that while the absorption of "L-tyrosine" and phenylalanine were complete (as shown by the presence of only scar tissue at the sites of injection), appreciable amounts of "DL-tyrosine" remained unabsorbed in Rats 1 and 2. The tyrosine recovered from these

<sup>3</sup> Synthesized and generously supplied by Drs. James Reid and Melvin Calvin, Radiation Laboratory, University of California. (See CALVIN *et al.*, *Isotopic Carbon*, Wiley, New York, 1949.)

rats and purified by isoelectric precipitation amounted to 80 mg. and 50 mg., respectively. The compound was optically inactive and had a radioactivity of 2320 counts/min./mg., showing that it was identical with the injected sample and that there had been no preferential absorption of one of the isomers. The quantities of the tyrosine and phenylalanine apparently absorbed by the rats are as follows: Rat 1, 160 mg.; Rat 2, 150 mg.; Rat 3, L- 224.2 mg., D- 0.8 mg.; Rat 4, L- 254.1 mg., D- 0.9 mg.; Rats 5 and 6, 125 mg.; Rats 7 and 8, L- 125 mg., L- 0.1 mg. The radioactivity of each of the absorbed doses of amino acids are shown in Table I.

TABLE II  
*Distribution of C<sup>14</sup> in the Urinary Glucose*

Compound	Counts/min./mmole		
	Glucose from "L-tyrosine"	Glucose from "DL-phenylalanine"	Glucose from "L-phenylalanine"
Phenylglucosotriazole (Carbons 1 to 6)	4,680	6,200	4,420
2-Phenyl-4-formylsotriazole (Carbons 1 to 3)	2,270	3,190	2,170
Formic acid (BaCO <sub>3</sub> ) (Carbons 4 and 5)	600 (1,200) <sup>a</sup>	—	—
Formaldehyde (dimedone) (Carbon 6)	1,130	1,500	1,070

<sup>a</sup> Figures in parenthesis give the activity for 2 mmoles; 1 mole of glucosotriazole gives 2 moles of formic acid.

The urine was analyzed for total radioactivity. Glucose, ketone bodies, and urea were isolated and assayed for C<sup>14</sup> as described previously (8). The results are given in Table I. The radioactive glucosotriazole samples obtained from the rats were degraded separately by periodate oxidation (8). These results are presented in Table II.

## DISCUSSION

The beta-carbon of both tyrosine and phenylalanine appeared in glucose and ketone bodies. The results with duplicate animals were in good agreement (Table I) except in the case of Rats 3 and 4. Rat 4 died 8 hr. after the injection; the urine contained blood indicating kidney damage which probably is responsible for the difference. It is interesting that, despite the toxic symptoms, the specific activity of glucose and ketone bodies were similar to those of Rat 3.

Comparison of the groups that received "DL-phenylalanine" and "L-phenylalanine" shows that the main differences are in the total C<sup>14</sup>

in urine (the latter about 55% lower) and in the specific activities of the excreted glucose ("L-phenylalanine about 30% lower). It is not possible to give a definite explanation of these differences from the data. The total  $C^{14}$  in glucose and in ketone bodies cannot be used as a basis for comparison when the difference is small since the amounts excreted by phlorizinized rats vary considerably.

In the case of rats that received tyrosine, the differences between total urinary activity and specific activity of glucose and ketone bodies are far greater (10-fold or more) and, interestingly enough, in the reverse order, the values with "L-tyrosine" being greater than with "DL-tyrosine." These differences cannot be due to differences in tyrosine absorption since, though the amino acid was administered as a suspension and part of the "DL-tyrosine" remained unabsorbed, the data in Table I are calculated for the amounts that had disappeared from the sites of injection. When labeled tryptophan, similarly diluted with DL- and L-tryptophan was administered, under identical conditions, the differences in excretory products were far less.<sup>4</sup> The behavior of tyrosine, therefore, appears to be quite unlike phenylalanine or tryptophan in this respect. Two possible explanations are: (a) the large D-tyrosine fraction in Rats 1 and 2 inhibited tyrosine metabolism; or (b) the trace of D-tyrosine with high specific activity in Rats 3 and 4 was metabolized more rapidly. The latter is unlikely especially since "L-tyrosine" values were of the same order of magnitude as "L-phenylalanine" and "L-tryptophan" values. Further experiments are required in order to determine the exact explanation.

Weinhouse and Millington (1) observed that acetoacetate labeled in the alpha-position was formed during the incubation of liver slices with tyrosine- $\beta$ - $C^{14}$ . A similar pathway of catabolism may be expected in phlorizinized rats. The methyl-labeled acetyl unit derived from the above acetoacetate would label Carbons 1, 2, 5, and 6 of glucose equally during its oxidation, *i.e.*, one-fourth of the glucose activity would be found in Carbon 6 (9). The results in Table II are in exact agreement with this and indicate the intermediary formation of a methyl-labeled acetyl derivative from beta-labeled tyrosine.

Similarly, the location of one-fourth of the glucose activity in Carbon 6 (Table II) is suggestive of the formation of a methyl-labeled acetyl derivative from beta-labeled phenylalanine. This conclusion is con-

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<sup>4</sup> Unpublished data.

sistent with the results of Schepartz and Gurin (2). They observed that the alpha-carbon of the side chain appears as the carboxyl of acetoacetate, and ring-carbon 1 or 3 appears as the terminal methyl group. In this scheme of degradation, the beta-carbon of the side chain may be expected to form the alpha-carbon of acetoacetate. Fission of the acetoacetate would yield a methyl-labeled acetyl group which, during oxidation, would label glucose in accordance with the observed results.

### SUMMARY

Small amounts of phenylalanine labeled with  $C^{14}$  on the beta-carbon of the side chain were mixed with DL- or L-phenylalanine and injected into phlorizinized rats. The glucose and ketone bodies isolated from the urine contained radioactivity. The total radioactivity in urine and the specific activity of glucose were slightly greater with DL-phenylalanine as the diluent than with L-phenylalanine.

In a similar study with beta-labeled tyrosine, the total urinary activity and the specific activities of the urinary glucose and ketone bodies were far greater (10-fold or more) with L-tyrosine as the diluent than with DL-tyrosine.

One-fourth of the radioactivity of the glucose derived from tyrosine as well as from phenylalanine was located in Carbon 6. It is concluded that the beta-carbons of the side chains of tyrosine and phenylalanine are converted to the alpha-carbon of acetate.

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# The Nucleic Acids of Plant Tissues. I. The Extraction and Estimation of Desoxypentose Nucleic Acid and Pentose Nucleic Acid <sup>1</sup>

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## INTRODUCTION

This report describes 1) a tissue fractionation procedure which can be paralleled cytochemically, 2) a differential acid treatment which extracts the constituents of pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) in separate fractions, 3) the application of spectrophotometric methods to the estimation of the nucleic acids in tissue extracts based on their ultraviolet absorption, and 4) the correlation of these results with the photolorimetric estimation of the pentose, desoxypentose, and phosphorus found in the extracts.

Most recent studies of the nucleic acid content of various animal tissues have been based upon the tissue fractionation methods developed by Schneider (1) and by Schmidt and Thannhauser (2). Reasonable agreement was found between the data obtained using either fractionation when assays were based upon pentose and desoxypentose estimations (3).

Various difficulties are encountered when one attempts to apply either of these methods to the study of the nucleic acids during the development of plant root tips or of pollen cells:

1) It is desirable in such studies to have methods capable of estimating approximately 1  $\mu$ g. of nucleic acid. The successful quantitative precipitation of DNA at this level in the Schmidt-Thannhauser separation is questionable.

2) Although the photolorimetric estimation of pentose and desoxypentose may be scaled successfully to this level employing microcuvettes in the Beckman spectrophotometer (4), preliminary attempts to apply the Schneider method to plant root

<sup>1</sup> This work was supported by a grant from the National Cancer Institute, U. S. Public Health Service; and by a grant from the American Cancer Society upon recommendation of the Committee on Growth.

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tips in this laboratory yielded very high pentose/phosphorus ratios and suggested that the orcinol reaction might be invalid as a measure of PNA in plant tissues due to interfering pentosans and polyuronides.

3) Although the high ultraviolet extinction of isolated nucleic acids may be applied to their quantitative estimation at levels of 1  $\mu\text{g.}$ , tissue extracts obtained with existing methods present certain obstacles. The Schmidt-Thannhauser alkaline extract contains interfering protein degradation products for ultraviolet measurements. The Schneider hot trichloroacetic acid extract presents several difficulties to ultraviolet examination, one of which is represented in Fig. 1. The absorption of solutions of trichloroacetic acid is significant at 260  $m\mu$  and increases sharply toward the shorter wavelengths. This interference of the trichloroacetic acid in the ultraviolet absorption of nucleic acid extracts has been circumvented by some workers (5) by diluting the trichloroacetic acid extract 10–100-fold and by using standards and blanks similarly treated. Such a dilution increases the quantity of nucleic acid necessary for an estimation to considerably more than 1  $\mu\text{g.}$

A possible substitute for trichloroacetic acid was suggested to us by Kalckar's (6) use of perchloric acid as a protein-precipitating extractant in the spectrophotometric estimation of mononucleotides. Figure 1

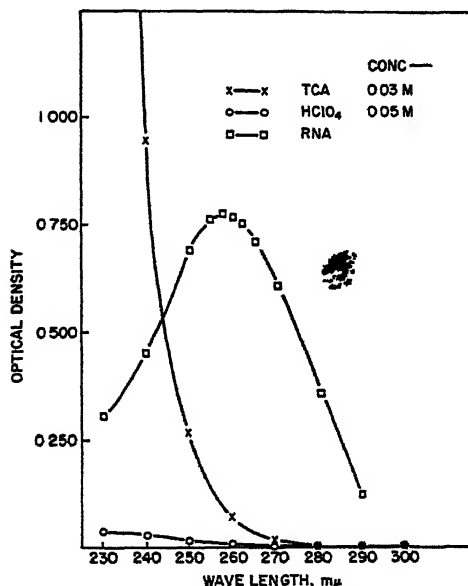


FIG. 1. The ultraviolet absorption of trichloroacetic acid and perchloric acid compared with that of ribonucleic acid. The concentrations of protein precipitants represented correspond to a 10-fold dilution of the levels used in extraction. Optical density =  $E = \log_{10} I_0/I$ .

indicates the negligible absorption of perchloric acid in the ultraviolet.

In exploring the conditions of time, temperature, and concentration for nucleic acid extraction with perchloric acid, it became apparent that PNA was more readily extracted than DNA.<sup>3</sup> Prolonged contact in the cold with 1 *N* perchloric acid slowly extracted the constituents of PNA but not DNA from tissue residues after prior removal of alcohol-soluble compounds, phospholipides, and acid-soluble compounds. Subsequent treatment with perchloric acid at elevated temperature extracted the constituents of DNA.

Differences in the hydrolytic extraction of PNA and DNA have been observed previously. The Schmidt-Thannhauser separation of PNA from DNA is based upon prolonged contact with alkali which degrades the PNA more rapidly than the DNA leaving the latter still precipitable with acid. Kaplan and Greenberg (7) have reported the splitting of rat liver nucleoprotein by cold trichloroacetic acid after long periods of contact (7 days). The successful demonstration of a nuclear apparatus in bacteria after acid hydrolysis has been interpreted (8) as due to the more rapid extraction of cytoplasmic constituents (including PNA) than nuclear constituents (DNA).

It seemed worth while, therefore, to attempt to work out the conditions for the reasonably sharp quantitative separation of PNA from DNA by differential acid extraction. Such a separation should be useful not only for assay purposes in simplifying calibrations and in the interpretation of analytical data, but also in isotopic tracer studies of differences in turnover rates in the two types of nucleic acids.

## EXPERIMENTAL

### *Assay Methods*

Phosphorus estimations were made according to the method of Fiske and SubbaRow (9) using the Klett-Summerson photocolormeter. 1-Amino-2-naphthol-4-sulfonic acid (Eastman Kodak Co.) was purified according to the procedure recommended (9).

Pentose estimations were made with several modifications of the reagent and procedure of Mejbaum (10) using the Klett-Summerson photocolormeter. The modified reagent used contained 0.033% instead of 0.1% of ferric chloride and has the advantage of producing smaller reagent blanks and somewhat increased sensitivity.

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\* In view of the well known acid insolubility of the intact nucleic acids it is apparent that only their degradation products are here being extracted. This situation applies as well to the Schneider (1) and to the Schmidt-Thannhauser (2) extractions. Such "extractions" although undesirable in a preparative sense, are useful for assay purposes if the constituents of the nucleic acids can be extracted completely and estimated.

Orcinol (Eastman Kodak Co.) yielded turbid solutions when heated with either the original or modified iron reagent unless the orcinol was first recrystallized twice from benzene. Orcinol (Mackay) produced no turbidity and was used without further purification. With the 45-min. heating period suggested by Albaum and Umbreit (11) we obtained a color equivalent to 2.4 moles of ribose for every 4 moles of pentose nucleic acid phosphorus (PNAP). It is possible to obtain a color equivalent to 2.0 moles of ribose for every 4 moles of PNAP by adjusting the heating time to 11 min. in a boiling water-bath. Although it is not true with any precision that one is measuring only purine bound pentose at the shorter heating time, as might be implied from the whole number factor, one positive advantage may be found in the tendency to reduce somewhat the interference of substances in tissue extracts which form furfural more slowly than does ribose. Under all conditions tried, however, we obtained high pentose/phosphorus ratios on nucleic acid extracts of plant tissues. On liver tissue extracts, satisfactory correspondence between pentose and phosphorus data was observed.

The diphenylamine reaction of Dische (12) was used as one measure of the DNA content of tissue extracts. Diphenylamine (Merck) was recrystallized from methanol. The blue color was developed by heating for 10 min. in a boiling water-bath, and after rapid cooling to room temperature, optical densities were determined immediately in the Beckman spectrophotometer. Maximum absorption was observed at 600  $m\mu$  with isolated samples and tissue extracts of DNA. Routinely, optical densities were also measured at several neighboring wavelengths to check the point of maximum absorption. In corn root tips a compound was observed yielding an intense purple color with an absorption peak at approximately 560  $m\mu$ , which would interfere seriously with DNA estimation. The interfering compound was however alcohol-soluble and was removed in the earliest tissue extract.

### *The Ultraviolet Absorption of Nucleic Acids*

Six samples of PNA isolated from yeast, 2 samples of DNA isolated from calf thymus, and 1 sample of DNA isolated from fish sperm were examined.<sup>4</sup> Aqueous solutions of these samples were prepared at a level of 50 mg./100 ml. and assayed using the analytical methods described above. Aliquots of these solutions were diluted in phosphate buffer at pH 7.4 for ultraviolet examination using the Beckman photoelectric quartz spectrophotometer.<sup>5</sup> These nucleic acids showed the characteristic absorption spectra in the ultraviolet. If the data at 280  $m\mu$  for yeast ribonucleic acid are calculated as extinction per mole of phosphorus (13),<sup>6</sup> a value of 9800 is obtained, identical with that reported by Chargaff for a purified commercial sample of yeast nucleic acid (14).

<sup>4</sup> We are indebted to Drs. K. G. Stern and D. O. Jordan for samples of thymus nucleic acid. Commercial samples of nucleic acids were obtained from Schwarz Laboratories, Eimer and Amend, General Biochemicals, Nutritional Biochemicals, Merck, and Pfanstiehl.

<sup>5</sup> We have found it more convenient in subsequent work at lower nucleic acid levels to read the ultraviolet absorption directly in 10% perchloric acid without pH adjustment or dilution in buffer.

<sup>6</sup>  $\mathcal{E}_P = 30.98 E/cl$ , where,  $E$  = extinction ( $\log I_0/I$ );  $c$  = P concentration, g./l.;

Treatment with perchloric acid, however, affects the ultraviolet absorption of the nucleic acids. Cold perchloric acid increases the extinction at 260  $m\mu$  of PNA and heating in perchloric acid shifts the point of maximum absorption from 260 to 268  $m\mu$  in the case of DNA. These effects are shown in Figs. 2 and 3. Calculations were therefore based on nucleic acid samples treated with perchloric acid in a manner similar to the extract to be assayed. Samples of nucleic acid of different origin differed significantly in extinction and in elementary composition but when their extinction was expressed in terms of their phosphorus

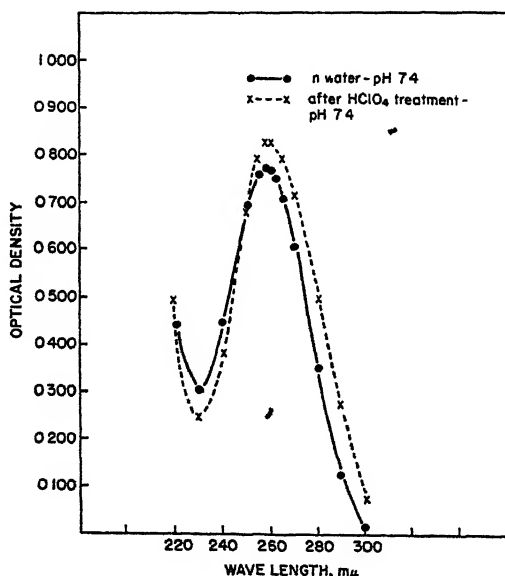


Fig. 2. The effect of cold perchloric acid treatment on the absorption spectrum of pentose nucleic acid.

content, agreement was quite good within each of the two types of nucleic acid, as recorded in Table I. It is thus possible to use commercially available, partially degraded samples of nucleic acid as standards in spectrophotometric assay provided that calculations are referred to nucleic acid phosphorus. Similar conclusions have been reached with respect to other photocolorimetric assays by various workers (1, 15).

On the basis of extinction per mole of phosphorus ( $\mathcal{E}_P$ ) one may also compare the ultraviolet absorption of perchloric acid extracts of

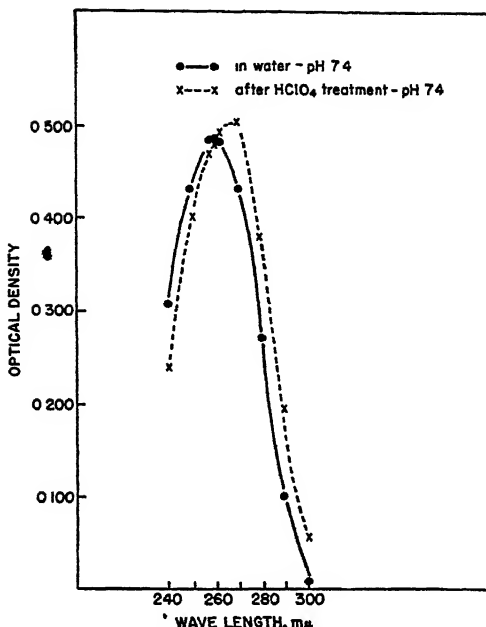


FIG. 3. The effect of hot perchloric acid treatment on the absorption spectrum of desoxypentose nucleic acid. The data shown above represent the effect of heating for 20 min. at 70°C. in 0.5 *N* perchloric acid.

tissues with that of isolated nucleic acids. Figure 4 represents the comparison of cold perchloric acid extracts of corn root tips and rabbit liver with a sample of yeast nucleic acid. The data shown will appear high compared to values previously reported unless it is recalled that perchloric acid treatment increases the extinction of PNA from an  $\epsilon_p$  of 9800 in water at pH 7.4 to an  $\epsilon_p$  of 10,800 after perchloric acid treatment and adjustment to pH 7.4.

### *Preparation of Plant Material*

Seeds of *Zea Mays*<sup>7</sup> were soaked overnight in distilled water and allowed to germinate in covered pyrex trays on moistened filter paper at 25°C. in the dark for 48–72 hr. Germination was not uniform and to reduce biological variability, seedlings were pooled according to the length of the primary root and stored in deep freeze. All plant data were obtained from apical 3 mm. segments of primary roots. This portion

<sup>7</sup> A commercially available double-cross hybrid seed corn (U. S. 13) was obtained from the Michell Seed Co., Philadelphia.

TABLE I  
*Ultraviolet Extinction of Nucleic Acid Samples in 1 N Perchloric Acid*

PNA			DNA		
sample	$E^a$ per $\mu\text{g.}$ P./ml.	$\mathcal{E}_P^b$	sample	$E$ per $\mu\text{g.}$ P./ml.	$\mathcal{E}_P$
Schwarz	0.352	10,880	Nutritional Biochemical	0.275	8,530
Eimer and Amend	0.354	10,920	Stern	0.291	9,020
Nutritional Biochemical	0.345	10,680	Jordan	0.283	8,780
General Biochemical	0.347	10,720			
Pfanstiehl	0.346	10,700			
Merck	0.357	11,000			
Mean	0.350	10,816	Mean	0.283	8,780
Standard deviation, $\%_c$		1.3	Standard deviation, $\%_c$		2.3

<sup>a</sup>  $E$  = Optical density =  $\log_{10} I_0/I$ .

<sup>b</sup>  $\mathcal{E}_P = 30.98 E/cl.$

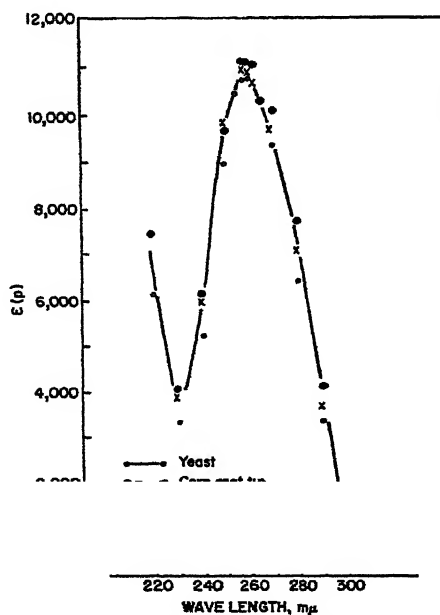


FIG. 4. The ultraviolet absorption of cold perchloric acid extracts of

pentose is observed and good agreement is obtained between ultraviolet, phosphorus, and pentose assays. No measurable amount of DNA is detected in the cold fractions by the diphenylamine reaction.

After 3 days of contact with cold perchloric acid, there was evidence that not only had the constituents of PNA been extracted from the tissue residue, but also a portion of the nitrogen bases of DNA.

Routinely, overnight contact in the cold for approximately 18 hr. is used to split the PNA from the tissue residue. The suspension is centrifuged and the residue is extracted twice more with 5 ml. portions of cold 1 *N* perchloric acid. The extracts are combined and made up to 15 ml. Three extractions are necessary, particularly where the PNA/DNA ratio is high. Here a holdback of a few per cent of the PNA fraction carried over into the DNA fraction introduces a significant error. This reveals itself in higher values of DNA calculated from the ultraviolet absorption and phosphorus content than from the diphenylamine reaction.

### *B. Extraction of DNA*

The residue from IIA is suspended in 5 ml. of 0.5 *N* perchloric acid and heated in a water-bath for 20 min. at 70°C. This process is repeated and the extracts are combined and made up to 10 ml. More exhaustive extraction of corn root tips failed to reveal any additional material reacting with diphenylamine.

## III. EXTRACTION OF ALKALI-SOLUBLE COMPOUNDS

The residue from IIB is suspended in 5 ml. of 2 *N* sodium hydroxide, heated in a boiling water-bath for 10 min., cooled, and centrifuged. The residue is resuspended in 5 ml. of 2 *N* hydrochloric acid and centrifuged. The extracts are combined and made up to 10 ml. This fraction, containing the constituents of phosphoprotein, comprised more than half of the total nitrogen and approximately 3% of the total phosphorus of the root tips studied.

### *Reproducibility of Assay Data*

Replicate runs with corn root-tip segments taken from roots of nearly comparable length and germination time are reproducible with a standard deviation of 10% for their PNA content, and 6% for their DNA content. In view of the uneven germination of corn seedlings, it seemed desirable to remove the problem of biological variability in determining the reproducibility of the assay method.

In the following experiment a single homogenate was prepared from 1000 3-mm. corn root-tip segments and made up to 11 ml. in alcohol. After vigorous agitation, 2 ml. aliquots were pipetted rapidly into centrifuge tubes and used for replicate runs. The data for the PNA fraction are shown in Table II. Ultraviolet data have been calculated in three ways based upon 1) the absorption at 280  $m\mu$ , 2) the absorption at 280  $m\mu$  minus that at 300  $m\mu$ , and 3) the absorption at 280  $m\mu$  minus that at 290  $m\mu$ . Calibrations for each procedure were determined by similar treatment of yeast nucleic acid. The essential agreement among the several methods of calculation and with the phosphorus data suggests the conclusion that the cold perchloric acid extract is relatively free of other substances absorbing in the ultraviolet. The procedure seems to be reproducible at this level with a standard deviation of about 2%.

**TABLE II**  
*Replicate Analyses for Pentose Nucleic Acid of Corn Root Tips  
 by Means of Cold Perchloric Acid Extraction Method*

Pentose nucleic acid phosphorus (PNAP) in $\mu\text{g./3-mm. segment}$				
Run no	PNAP (calculated from U.V. data)			PNAP (found)
	260 $m\mu$	260-300 $m\mu$	260-290 $m\mu$	
32	1.86	1.86	1.76	1.81
33	1.81	1.85	1.74	1.81
34	1.82	1.85	1.76	1.78
35	1.76	1.76	1.68	1.74
Mean	1.81	1.83	1.74	1.79
Standard deviation, %	1.9	2.1	1.8	1.8

The data for the DNA fraction are shown in Table III. Here we find good agreement between three independent assays. The ultraviolet absorption is accounted for by the desoxypentose and phosphorus content of the fraction with a standard deviation of a few per cent and replicate runs are reproducible to about the same extent.

**TABLE III**  
*Replicate Analyses for Desoxypentose Nucleic Acid of Corn Root Tips  
 by Means of Hot Perchloric Acid Extraction Method*

Desoxypentose nucleic acid phosphorus (DNAP) in $\mu\text{g./3-mm. segment}$				
Run no.	DNAP (from U.V. data)		DNAP (found)	DNAP (diphenylamine)
	260 $m\mu$	270 $m\mu$		
32	0.299	0.296	0.302	0.292
33	0.307	0.307	0.308	0.319
34	0.296	0.300	0.295	0.324
Mean	0.301	0.301	0.302	0.312
Standard deviation, %	1.5	1.5	1.7	4.5

Attempts to determine the nitrogen content of perchloric acid extracts by digestion and Nesslerization yield unreliable and generally low results. Similar experience has been recorded (17) where perchloric acid was added to speed micro-Kjeldahl digestion and the loss of nitrogen through decomposition of ammonium perchlorate is suggested. We are therefore unable to report any meaningful nitrogen data on the composition of the nucleic acid fractions.

Several points may be made with respect to the completeness of extraction into each of the nucleic acid fractions. To test whether or not the contact with cold 0.2 *N* perchloric acid removed any PNA, one of the 2.0 ml. aliquots of the large root-tip homogenate used in the replicate runs of Table II was treated with 3 portions of 0.2 *N* perchloric acid for 1 hr. each in the cold and then carried through treatment with 1 *N* perchloric acid overnight. No absorption peak at 260  $m\mu$  was observed in the 0.2 *N* perchloric acid fractions, and 1.80  $\mu$ g. of PNAP/3-mm. segment was recovered in the 1 *N* perchloric acid fraction. This agrees closely with the mean of the quadruplicate runs reported in Table II. The completeness of extraction of PNA by cold 1 *N* perchloric acid is supported by the results of the repeated extraction represented in the time curves of Fig. 5 and by the agreement of the independent assay methods on the DNA fraction. Any carry-over of unextracted PNA into the DNA fraction would be revealed by higher values of DNAP calculated from the ultraviolet and phosphorus data than from the diphenylamine reaction. Completeness of extraction of DNA is supported by the observation that exhaustive extraction with perchloric acid at higher concentration and temperature fails to extract any additional amounts of DNA.

#### *The Nucleic Acid Content of the Corn Root Tip Meristem*

In Table IV is recorded the nucleic acid content of the corn root tip meristem on several bases both as nucleic acid phosphorus and as nucleic acid. PNAP was converted to PNA and DNAP to DNA by dividing by 0.099 and 0.095, respectively, for ease of comparison with data on other tissues reported by previous workers. These factors, taken from Schneider (1), appear to be derived from an assumed tetranucleotide composition.

Calculations of the nucleic acid content on a per cell basis have been made with a cell number estimate provided for us by Dr. R. O. Erickson and Mrs. K. B. Sax.

TABLE IV  
*The Nucleic Acid Content of the Corn Root Tip Meristem*

	$\mu\text{g./3-mm.}$ segment	$\mu\text{g./mg.}$ fresh weight <sup>a</sup>	$\mu\text{g./mg.}$ dry weight <sup>b</sup>	mg./cell $\times 10^6$
PNAP	1.81	0.794	4.42	—
PNA	19.0	8.35	46.3	93
DNAP	0.301	0.132	0.735	—
DNA	3.04	1.33	7.42	15

<sup>a</sup> Mean fresh weight data used for the calculation of the results in Table IV were obtained by weighing 100 apical 3-mm. corn root-tip segments on a Roller Smith torsion balance in replicate runs.

<sup>b</sup> Mean dry weight data were obtained after drying at 60°C. for 48 hr. in a forced draft oven.

Techniques for counting all the cells of such a structure with high precision are not yet available, but in view of the growing interest in the DNA content on a per cell basis pointed out to us by Dr. Alfred Mirsky, it seemed desirable to attempt to fix the order of magnitude of this quantity for corn roots in spite of several approximations inherent in the cell count.

#### *Comparison of Perchloric Acid and Trichloroacetic Acid and Extractions*

Before adopting a new method of fractionation and assay it seems desirable to compare it with some method in current use to see whether the same things are being estimated. Since corn root-tip tissue presents obstacles to the Schneider method which are the basis of this study,

TABLE V  
*Comparison of Perchloric Acid and Trichloroacetic Acid  
 Extractions of Rabbit Liver*

	Mg./100 mg. wet tissue					
	PNAP			DNAP		
	U.V. data at 260 m $\mu$	Orcinol reaction	P found	U.V. data at 260 m $\mu$	Diphenyl- amine reaction	P found
Perchloric acid method	32.8	35.4	31.9	14.1	14.1	16.1
Trichloroacetic acid method		34.9			14.8	

the comparison must be made on comparable samples of an animal tissue.

A rabbit-liver homogenate was prepared in alcohol, and, after vigorous shaking, aliquots were pipetted into each of several tubes which were used in comparative experiments. With the rabbit liver both PNA and DNA were extracted more slowly by means of perchloric acid than from the corn root tip using the same conditions. Since an 18-hr. cold extraction was still adequate for complete PNA extraction, only the hot procedure was modified by increasing the concentration of perchloric acid to 1 *N*, the temperature to 80°C., and the time to 30 min. in each of two extractions. A parallel run was made according to the Schneider procedure. Results are shown in Table V. These data, showing essential agreement between the two procedures, are offered only for the purpose of comparison in view of our lack of information about the age, sex, and nutritional state of the animal from which the liver was obtained.

#### ACKNOWLEDGMENTS

The authors are grateful for the many suggestions and the continued interest of Drs. D. R. Goddard, R. O. Erickson and H. G. Albaum.

#### SUMMARY

1. The ultraviolet absorption of several isolated samples of nucleic acid from yeast, thymus, and fish sperm, and of extracts of corn root tips and rabbit liver are compared, based on their phosphorus content.

2. A tissue fractionation method is proposed for nucleic acid assay which is based upon a differential acid extraction of PNA and DNA into separate fractions. This method may also be applied cytochemically in parallel studies.

3. Perchloric acid, a protein-precipitating acid with no significant ultraviolet absorption, has been applied to the extraction and spectrophotometric assay of nucleic acids at microgram levels.

4. Good agreement has been observed in several independent assays of nucleic acid in perchloric acid extracts of corn root tips and rabbit liver.

5. Essential agreement has been observed between the proposed method and the Schneider method on rabbit liver.

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# The Occurrence of Quercetin in the Pollen of *Zea Mays*<sup>1</sup>

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## INTRODUCTION

Pollen from corn is readily obtainable (1); however, the exact nature of its pigments has remained unknown. Vinson (2) obtained a yellow crystalline substance which he did not identify. Uber (3), in further studies, was unable to correlate the absorption spectrum with the chemical composition. Since the yellow pigment from the ether extract of corn pollen has not been identified, a method for the separation and characterization of quercetin is described. It was obtained in almost equal yields from the pollen of *Zea mays*, varieties *Golden Cross Bantam* and *Ohio M-15*.

## EXPERIMENTAL

### *Extraction of Pollen*

Two kg. of freshly collected corn pollen was placed in a 5-l. flask. The pollen was covered with peroxide-free ether and permitted to stand for a day at room temperature. Periodically during extraction the ether was kneaded into the dough-like mass of pollen. The extract was then decanted and replaced with fresh ether, and this extraction and decantation was subsequently repeated at daily intervals until 10 fresh portions of ether had been used for each flask of pollen. The combined ether extracts were concentrated by distilling off the ether, the last traces of solvent being removed by evacuating the flask containing the extract. From 2 kg. of pollen was obtained 61 g. of ether-soluble oil.

### *Purification of Pigment*

Fifty g. of the ether-soluble oil was stirred into 250 ml. of petroleum ether (b. p. 35–60°), and the flask containing the suspension of yellow pigment was then permitted

<sup>1</sup> Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 1074 (n.s.).

to stand at 4° for 24 hr. The pigment was then collected and washed with 50 ml. of petroleum ether. The yield was 2.25 g. The entire quantity of crude pigment thus obtained was dissolved in 100 ml. of boiling 95% ethanol, and filtered. To the filtrate was added dropwise a hot solution of 10 g. of neutral lead acetate in 25 ml. of 95% alcohol, followed by enough 12 *N* sodium hydroxide to adjust the solution to a pH of 8-9.

After the alcoholic suspension of lead salt had cooled to room temperature, the orange-colored solid was filtered off and washed with 25 ml. of 95% ethanol. When the filter cake was dry, it was dissolved in 25 ml. of boiling glacial acetic acid, and the solution diluted with 75 ml. of water. Upon cooling, lemon yellow crystals formed. These were filtered off, recrystallized 3 times from 50% aqueous acetone and dried over phosphorus pentoxide in a vacuum at 90° for 72 hr. *M. p.* 312-15° (dec.). The reported *m. p.* of quercetin is 313-14° (dec.) (4).

*Anal.* Calcd. for  $C_{15}H_{10}O_7$ : C, 59.6%; H, 3.3%; *Mol. wt.*, 302. Found: C, 59.7%; H, 3.6%; *Mol. wt.* (Rast, in camphor), 288.

### Acetylation of Pigment

Five hundred and ten mg. of the purified pigment was refluxed with 10 ml. of acetic anhydride and 5 mg. of concentrated sulfuric acid for 1.5 hr. The reaction mixture was then poured into 20 ml. of methanol, and the volatile components evaporated *in vacuo*. The residue was recrystallized 3 times from hot 95% ethanol. Decolorizing carbon was added during the first recrystallization. The yield was 150 mg. of colorless silky needles having a *m. p.* of 199-99.5° after vacuum drying over phosphorus pentoxide at 90°. The reported *m. p.* of quercetin pentacetate is 200° (5). The acetylated product showed no melting point depression when mixed with an authentic sample of quercetin pentacetate.

*Anal.* Calcd. for quercetin pentacetate,  $C_{25}H_{30}O_{13}$ : C, 58.6%; H, 3.9%; Acetyl, 42.0%; *Mol. wt.*, 512. Found: C, 58.4%; H, 4.1%; Acetyl (from C-Methyl det'n.), 41.9%; *Mol. wt.* (Rast, in camphor), 530.

### Methylation of Pigment

Five hundred mg. of purified pigment was dissolved in 60 ml. of acetone. To this solution were added 6 ml. of water, 5 ml. of dimethyl sulfate, and 6 g. of powdered sodium carbonate monohydrate. The mixture was refluxed for 1.25 hr., cooled, and poured into 100 ml. of water. After standing at 4° for 1 hr., the slurry was filtered on a suction filter. The filter cake (350 mg.) was recrystallized 3 times from hot 75% acetone. Decolorizing carbon was added during the first recrystallization.

The product melted at 161-61.5° following drying over phosphorus pentoxide at 90° in a vacuum. The reported *m. p.* of quercetin 3,7,3',4'-tetramethyl ether is 159-60° (6).

No depression of melting point was noted with an authentic sample of quercetin 3,7,3',4'-tetramethyl ether.

*Anal.* Calcd. for quercetin tetramethyl ether,  $C_{18}H_{18}O_7$ : C, 63.7%; H, 5.1%; Methoxyl, 34.6%; *Mol. wt.*, 358. Found: C, 63.5%; H, 5.3%; Methoxyl, 34.5%; *Mol. wt.* (Rast, in camphor), 349.

## SUMMARY

The yellow pigment of corn (*Zea mays*) pollen has been identified as quercetin. It was isolated from the pollen of *Zea mays*, varieties, *Golden Cross Bantam* and *Ohio M-15*. Quercetin was characterized as its pentacetate and as its 3,7,3',4'-tetramethyl ether, for which a new method of preparation is described.

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# Use of C<sup>14</sup> in the Study of the Acid Metabolism of *Bryophyllum calycinum*<sup>1</sup>

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## INTRODUCTION

The acid metabolism of succulents, of which *Bryophyllum calycinum* is a typical member, is different from that of other acid-forming species in that the acids do not merely accumulate, but rather undergo daily variations in concentration. Since the early experiments of Mayer (1) it has been known that in the light, succulents produce carbohydrates from the organic acids previously formed in the dark. In addition to the deacidification which occurs in the light, a deacidification also occurs in prolonged darkness. Bennet-Clark (2) postulated that this deacidification in the dark is an enzymatic dismutation in which part of the acids is reduced to carbohydrates and a part simultaneously oxidized to carbon dioxide. Of the organic acids present in *Bryophyllum*, malic acid has been found to play the most important role in the acid  $\rightleftharpoons$  carbohydrate transformations (3).

The experimental work described in this paper was undertaken in an effort to provide answers to three specific questions. (a) Is the deacidification of succulents in light a direct reduction of organic acids to carbohydrates or an oxidation of these acids to carbon dioxide followed by reduction of the carbon dioxide to carbohydrates? (b) If the deacidification is the result of a direct reduction, what are the intermediates between the organic acids and the carbohydrates? (c) Are carbohydrates formed from organic acids during deacidification occurring in the dark?

<sup>1</sup> The data presented are taken from a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Agricultural Biochemistry, The Ohio State University, Columbus, Ohio.

## EXPERIMENTAL

In order to answer the above questions the following experiments were performed:

I. Detached leaves of *Bryophyllum calycinum* were exposed to an atmosphere containing  $C^{14}O_2$  in the dark at  $10^\circ C$ . for definite periods of time. This temperature was chosen because Pucher *et al.* (3) found it favorable to a rapid production of organic acids in *Bryophyllum*.

TABLE I  
*Distribution of Activity in the Leaf Tissue*  
(In terms of per cent of the total count)

Experiment	Organic acids	EtOH extract	Soluble sugars	Starch
I				
Dark 2 hr. at $10^\circ C$ .	84.5	15.0	0.4	0.1
IIa				
Dark 4 hr. at $10^\circ C$ . Light 1 hr. at $13^\circ C$ .	48.6	16.2	7.3	28.2
IIb				
Dark 2.5 hr. at $10^\circ C$ . Light 0.5 hr. at $13^\circ C$ .	87.9	7.2	1.4	3.7
III				
Light 0.5 hr. at $13^\circ C$ .	1.1	24.2	15.6	59.0
IV				
Dark 4 hr. at $10^\circ C$ . Dark 24 hr. at $25^\circ C$ .	94.5	5.5	0.3	0.03

Organic acids, sugars, and starch were isolated from these leaves and assayed for radioactive carbon. Malic and citric acids were separated, degraded and the  $C^{14}$  labeling determined.

II. Comparable leaves were exposed in the dark to  $C^{14}O_2$  after which they were exposed to light for a short period of time in an atmosphere free of  $C^{14}O_2$ . From these leaves organic acids, sugars, and starch were isolated, assayed for radioactive carbon, and the results compared with those of the preceding experiment. Malic acid, sugars, and starch were separated, degraded, and the  $C^{14}$  labeling determined.

III. Detached *Bryophyllum* leaves were exposed to an atmosphere containing  $C^{14}O_2$  and to light simultaneously for a short period of time. The organic acids and carbohydrates were isolated and assayed for radioactivity. The sugars and starch were degraded and the labeling determined. These results were compared with those of the preceding experiments.

IV. Detached *Bryophyllum* leaves were exposed to an atmosphere containing  $C^{14}O_2$  in the dark for a few hours at  $10^\circ C$ . The excess  $C^{14}O_2$  was removed and the leaves allowed to remain in the dark at room temperature for 24 hr. The organic acids and carbohydrates were isolated and assayed for radioactivity.

### METHODS

The leaves were exposed to an atmosphere of 5%  $CO_2$  containing  $C^{14}O_2$  (5) in a reaction chamber similar to that used by Putnam *et al.* (6). After exposure the leaves

TABLE II  
*Uptake of Labeled Carbon Dioxide*

Experiment	$C^{14}O_2$ uptake /100 mg. of organic solids mg.	$C^{14}O_2$ uptake /100 mg. of malic acid mg.
Ia		
Dark 2 hr. at $10^\circ C$ .	0.06	0.6
Ib		
Dark 2.5 hr. at $10^\circ C$ .	0.08	6
Ic		
Dark 4 hr. at $10^\circ C$ .	0.4	24
IIa		
Dark 4 hr. at $10^\circ C$ .		
Light 1 hr. at $13^\circ C$ .	0.2	8
IIb		
Dark 2.5 hr. at $10^\circ C$ .		
Light 0.5 hr. at $13^\circ C$ .	0.15	3
III		
Light 0.5 hr. at $13^\circ C$ .	0.3	—
IV		
Dark 4 hr. at $10^\circ C$ .		
Dark 24 hr. at $25^\circ C$ .	0.2	10

were immediately frozen with solid  $\text{CO}_2$ , ground to a fine powder, and lyophilized. The dried sample was then moistened with dilute  $\text{H}_2\text{SO}_4$  and extracted with alcohol-free ether. The organic acids were separated from this extract by partition chromatography on a silica gel column (7) and the amounts determined by titration. Pucher and Vickery (4, 8) have shown that *Bryophyllum* leaves contain five organic acids, viz. succinic, oxalic, malic, citric, and isocitric. The partition coefficients of four of the five organic acid fractions actually obtained by chromatographic separation corresponded to the partition coefficients of authentic samples of succinic, oxalic, malic,

TABLE III  
*Distribution of Activity in the Organic Acids*  
(In terms of per cent of total count for the organic acids)

Experiment	Succinic	Oxalic	Malic	Citric	Isocitric
Ia					
Dark 2 hr. at 10°C.	1.8	0.8	76	20	1.4
Ib					
Dark 2.5 hr. at 10°C.	3.0	0.2	80	15.2	1.4
Ic					
Dark 4 hr. at 10°C.	5.5	0.2	81	11.7	2.0
II					
Dark 4 hr. at 10°C. Light 1 hr. at 13°C.	1.1	3.1	62	30	4.3
III					
Light 0.5 hr. at 13°C.	40	13.2	19.8	21	5.9
IV					
Dark 4 hr. at 10°C. Dark 24 hr. at 25°C.	5.3	11.0	20	60	2.3

and citric acids. The fifth fraction was very probably isocitric acid (4) but this could not be verified because of lack of a pure sample. The citric acid was degraded by the method of Weinhouse (9). Malic acid was degraded according to the method of Wood (10); and the carbon of the 1-carboxyl group was removed as carbon monoxide according to the procedure of Pechmann<sup>2</sup> (11). The CO was then converted to  $\text{CO}_2$  by oxidation with  $\text{I}_2\text{O}_5$ .

The residue from the ether extraction was further extracted with 80% ethyl alcohol. After removal of the alcohol, the amino acids were separated from the sugars by adsorption on ion-exchange resins. The sugar solution was concentrated, an aliquot

<sup>2</sup> This method has not been checked with synthetic  $\text{C}_1$  carboxyl-labeled malic acid.

TABLE IV  
*Distribution of Activity in the Organic Acids*  
 (In terms of specific activities. Malic acid = 100)

Experiment	Succinic	Oxalic	Malic	Citric	Isocitric
Ia Dark 2 hr. at 10°C.	35	1.6	100	54	1.6
Ib Dark 2.5 hr. at 10°C.	21	2.3	100	29	1.1
Ic Dark 4 hr. at 10°C.	28	0.7	100	18	0.7
II Dark 4 hr. at 10°C. Light 1 hr. at 13°C.	12	1.5	100	32	2.7
III Light 0.5 hr. at 13°C.	192	27	100	122	32
IV Dark 4 hr. at 10°C. Dark 24 hr. at 25°C.	60	12	100	140	22

taken for radioactive assay, and the remainder used in degradation procedures according to the method of Wood (12).

Starch was isolated from the residue from the alcoholic extraction by the perchloric acid gelatinization method (13). After hydrolysis, the glucose was degraded according to the above mentioned procedure (12).

To determine the distribution of radioactivity among the compounds isolated, aliquots of the various samples were prepared for counting. Organic acids were counted as the sodium salts. Carbohydrates were counted as such. In the degradation studies all counts were made on barium carbonate.<sup>3</sup> For counting, the Radiation Counter Laboratories' Nucleometer, Mark 9, Model 1, Serial B-35 was used.<sup>4</sup> The organic compounds were mounted as very thin layers (< 1 mg./cm.<sup>2</sup>). The barium carbonate was mounted at or near infinite thickness.

## RESULTS AND DISCUSSION

The results of these experiments can best be expressed in the form of Tables.

<sup>3</sup> The BaC<sup>14</sup>O<sub>3</sub> used in this investigation was supplied by The Oak Ridge National Laboratory.

<sup>4</sup> Made available by Dr. Marion Pool of the Department of Physics.

From Table I, Expt. I, it is apparent that most of the  $C^{14}O_2$  that is fixed by *Bryophyllum* in the dark is incorporated in the organic acids. Similar results have been obtained by Thurlow and Bonner (14). In the light (Expt. III), only a relatively small amount of the  $C^{14}O_2$  appears in organic acids. The accepted view that starch can be formed in the light from organic acids is confirmed (Expt. II). No evidence

TABLE V  
*Distribution of Labeling in the Active Compounds*  
(In terms of per cent of  $C^{14}$  in the various positions)

Compound degraded	Ia Dark 2 hr. at 10°C.	Ib Dark 2.5 hr. at 10°C.	Ic Dark 4 hr. at 10°C.	IIa Dark 4 hr. at 10°C. Light 1 hr. at 13°C.	IIb Dark 2.5 hr. at 10°C. Light 0.5 hr. at 13°C.	III Light 0.5 hr. at 13°C.
Malic acid —CH <sub>2</sub> —CHOH—	4	8	8	21	—	—
1-COOH	—	38	—	34	—	—
4-COOH	—	54	—	45	—	—
Citric acid						
6-COOH	—	—	51	—	—	—
1,5 COOH's	—	—	42	—	—	—
Soluble hexoses						
3,4 C atoms	—	—	—	49	—	40
2,5 C atoms	—	—	—	—	—	—
				51	—	60
1,6 C atoms	—	—	—	—	—	—
Glucose from starch						
3,4 C atoms	—	—	—	52	53	45
2,5 C atoms	—	—	—	—	29	34
				48	—	—
1,6 C atoms	—	—	—	—	19	21

could be found for the idea that starch can be formed in the dark from organic acids (Expt. IV). See Pucher *et al.* (4).

It should be emphasized that in these experiments, changes of the recently assimilated  $C^{14}O_2$  are being studied rather than changes in the organic solids already present in the leaves. Table II shows the relationship between the quantities of  $C^{14}O_2$  assimilated and the total

organic solids; and between the  $C^{14}O_2$  assimilated and the total malic acid content of the leaves.

From Tables III and IV confirmation is given to previous observations that malic is the chief acid concerned in the acidification of *Bryophyllum* leaves which occurs in the dark (Expt. Ia, b, and c). In Expt. IV, a redistribution of activity among the organic acids occurs. From Table IV a comparison of Expts. I and II shows that when exposed to light the specific activity of succinic relative to malic acid decreases. If succinic were an intermediate (15) in the conversion of malic acid to carbohydrate, the reverse might be expected.

From Table V, the preferential labeling of the carboxyl groups of malic acid lends support to the suggestion of Bonner and Bonner (5) that it is formed by the Wood and Werkman reaction. Citric acid appears to be formed by carboxylation (Expt. Ic). In hexose synthesis from malic acid by *Bryophyllum*, it might be expected that the 2, 5 positions would be most heavily labeled if the mechanism suggested by Calvin and Benson (15) were operative. However, the heaviest labeling occurs in the 3, 4 positions (Expt. IIa and b). A reversal of the Wood and Werkman reaction would account for the labeling found.

#### SUMMARY

1. A reversal of the Wood and Werkman reaction seems a more probable explanation of the deacidification of *Bryophyllum* in the light than either a direct reduction of malic acid to carbohydrate or an oxidation of the malic acid to carbon dioxide followed by carbohydrate formation.

2. Under the conditions of these experiments, no appreciable carbohydrate synthesis from organic acids occurs during deacidification of *Bryophyllum* leaves in the dark.

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# The Bacterial Metabolism of Glycine <sup>1</sup>

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## INTRODUCTION

The mechanism of the bacterial oxidative dissimilation of glycine will be discussed in the present contribution. Oxidation of glycine by a species of *Achromobacter* has been shown previously (1). The R. Q. of the dissimilation was 1.5; one mole of ammonia was formed and one mole of oxygen was utilized for each mole of glycine oxidized. The data indicated that the oxidation of glycine did not proceed to completion but that some unidentified one-carbon compound, or its homolog, was formed.

## METHODS

The organism employed was the same strain of *Achromobacter* used previously (1). Nonproliferating bacterial suspensions were prepared by inoculating nutrient agar in Roux flasks and harvesting the cells after incubation for 24 hr. at 30°C. The bacteria were washed 3 times and stored in the refrigerator; the cells were again washed immediately before use and suspended in distilled water.

Glycine was determined according to Alexander *et al.* (2). Ammonia was determined colorimetrically by nesslerizing the distillate from an alkaline deproteinized sample of liquor. An all-glass micro-Kjeldahl apparatus was employed. Oxygen uptake and carbon dioxide production were determined manometrically. Formaldehyde was determined by procedures similar to those used for glycine except that decarboxylation by ninhydrin was omitted. Formic acid was determined titrimetrically (3). Methylamine was detected by distilling an alkaline solution of the deproteinized protein liquor into a 0.5% alcoholic solution of 2,4-dinitrochlorobenzene. In the presence of methylamine, a precipitate of 2,4-dinitromethylaniline forms within 24 hr. Interfering ammonia was removed prior to distillation by absorption with yellow mercuric oxide (4). Hydrogen peroxide as a metabolic intermediate was detected manometrically (5). The presence of peroxidase was determined by oxidation of pyrogallol (6). Pentose was determined both qualitatively and quantitatively by the

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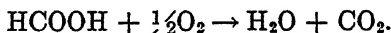
<sup>1</sup> Journal Paper No. 1433 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 975.

Mejbaum modification of Bial's orcinol test (7). Confirmatory tests for carbohydrates were made by adding 3 drops of a 5% alcoholic solution of thymol to a sample of the deproteinized fermentation liquor and layering the suspension on concentrated sulfuric acid. Carbohydrates develop a red color at the interface. Since aldehydes, formic acid, lactic acid, oxalic acid, citric acid, and acetone interfere, the test was used in conjunction with the orcinol reaction. Reducing carbohydrates in bacterial hydrolyzates were determined by the method of Stiles *et al.* (8). An aliquot of the bacterial suspension was hydrolyzed for 3 hr. with 5 mmoles of HCl/g. of bacteria. Phenylhydrazine derivatives were prepared according to Van der Haar (9). Derivatives of 2,4-dinitrophenylhydrazine were prepared by reacting a saturated solution of the hydrochloride with a deproteinized sample of fermentation liquor in a boiling water bath for 30 min. Formaldimedone derivatives were prepared by adding a solution of the reagent to a solution of glycine being oxidized by the bacteria. Formaldimedone was precipitated from acid solution and was recrystallized from a minimum volume of hot glacial acetic acid or methyl cellosolve. Periodic acid was prepared by dissolving 5.75 g. of  $\text{KIO}_4$  in 100 ml. of 3.6 *N*  $\text{H}_2\text{SO}_4$ .

### EXPERIMENTAL

Ten ml. of a 6.5% suspension of nonproliferating *Achromobacter* cells were added to a 125 ml. Warburg flask containing 1 mmole of glycine and 10 ml. of  $M/15$  phosphate buffer at pH 6.7. A control flask containing buffer but no glycine was inoculated similarly. The flasks were incubated with shaking at  $30.4^\circ$  for 14.5 hr. Oxidation of glycine was determined manometrically; cup contents were removed for analysis when the oxygen uptakes of the cells in both flasks were approximately equal. One and three-tenths mmoles of  $\text{NH}_3$  were formed from 1 mmole of glycine. Qualitative tests for methylamine, methyl alcohol, formaldehyde, and acetic acid were negative, but a trace of formic acid was detected. Nonproliferating cells produced approximately  $6.2 \times 10^{-4}$  mmoles of formic acid from 0.3 mmoles of glycine; an average of 0.17 mmoles of formic acid corresponded to the oxidation of 51 mmoles of glycine.

Traces of formic acid indicated that it might be an intermediate in glycine oxidation. Sodium formate was incubated with nonproliferating cells and one-half mole of oxygen was utilized for each mole of formate oxidized, and 1 mole of  $\text{CO}_2$  was formed (Table I). The data indicate that formic acid is oxidized according to the equation:



Glyoxylic acid has been identified as a product of glycine oxidation (10, 11). Attempts were made to isolate glyoxylic acid during the oxidation of glycine by adding the keto-fixatives hydroxylamine, semicarbazide, *m*-nitrophenylhydrazine, phenylhydrazine, sodium bisulfite, and dimedon.

It was expected that if the fixation of an intermediate occurred, it could be detected manometrically. Sodium bisulfite was found to be too variable in its effect to warrant its use. The action of the fixatives is of interest in that in almost all cases their addition to cells incubated with glycine resulted in a decrease in oxygen uptake to a value below that of the residual or endogenous respiration. One hundred per cent inhibition was obtained with 0.02 *M* and 0.01 *M* hydroxylamine, 0.02 *M* semicarbazide, 0.004 *M* *m*-nitrophenylhydrazine, and 0.004 *M* phenylhydrazine. On the other hand, when hydroxylamine was added to *Achromobacter* incubated with glucose, there was almost no poisoning effect; 314 ml. O<sub>2</sub> were taken up as compared with 321 ml. by cells incubated with glucose in the absence of hydroxylamine. The inhibition of oxidation by fixatives is, therefore, not a general poisoning.

TABLE I

*Oxidation of Formate by Achromobacter*

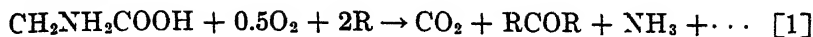
Each flask contained 0.5 ml. of 10% cell suspension, 1.0 ml. of *M*/15 phosphate buffer, pH 6.8, and 0.5 ml. substrate. Figures corrected for endogenous respiration. Flask 1, time = 180 min., flask 2, time = 450 min. Temperature = 30.4°C.

Expt. no.	Formate	Oxygen utilized	Carbon dioxide produced	Oxygen $\mu$ moles		Carbon dioxide, $\mu$ moles		R Q	
				Found	Theory	Found	Theory	Found	Theory
1	$\mu$ moles 20.0	$\mu$ l. 213	$\mu$ l 414	9.5	10.0	18.5	20.0	1.95	2.0
2	29.5	302	649	13.5	14.8	29.0	29.5	2.14	2.0

Dimedon decreased the oxygen uptake of cells by about 50%. This effect was not always found, but cells stored in the refrigerator for 2 weeks were more susceptible to the action of dimedon than were freshly harvested cells. Dimedon solutions were adjusted to pH 6.8–7.0 before use.

Manometric studies were made of cells incubated with glycine and dimedon. Table II shows that whereas the fixative did not affect the deamination of glycine, the R. Q. changed from 1.5 to 2.0, and the oxygen uptake was decreased from 1 mole to one-half mole/mole of

glycine. These changes may be represented by equation [1] where R is dimedon:



$$\text{R. Q.} = \frac{1}{0.5} = 2.0$$

This equation may represent the initial oxidation of glycine to glyoxylic acid, and subsequent decarboxylation of glyoxylic acid to formaldehyde which could react with dimedon. The equation would also be obtained if the glycine were oxidized to glyoxylic acid and this reacted with the dimedon to give a product which on decarboxylation would be the same as formaldimedone. A glyoxylate derivate prepared by reacting dimedon with glyoxylic acid<sup>2</sup> and recrystallized

TABLE II

*The Effect of Dimedon on the Oxidation of Glycine*

Each cup contained 0.5 ml. of 10% *Achromobacter* suspension, 0.5 ml. of 0.1 M phosphate buffer, pH 6.8, 0.5 ml. of 0.04 M glycine, and 0.5 ml. dimedon. Figures corrected for endogenous respiration. Final volume = 2.0 ml. Time = 390 min., temperature = 30.4°C.

Dimedon	Glycine	Oxygen utilized	Carbon dioxide produced	Oxygen	Carbon dioxide	NH <sub>3</sub>	R. Q.	$\frac{\text{NH}_3}{\text{Glycine}}$
<i>moles</i>	<i>μ moles</i>	<i>μl</i>	<i>μl</i>	<i>μ moles</i>	<i>μ moles</i>	<i>μ moles</i>		
0	20.0	435	655	19.4	29.3	18.5	1.51	0.93
0.04	20.0	208	413	9.3	18.4	19.0	1.99	0.95

from hot glacial acetic acid melted at 239°C. Nonproliferating cells were incubated with this derivative in Warburg flasks but no evidence of decarboxylation was found, suggesting that a product of glyoxylic acid reacts with dimedon rather than glyoxylic acid itself.

It is reasonable to assume the presence of glyoxylic acid as an intermediate. Other possible oxidation products are glycolic acid, methyl alcohol or methylamine, and subsequent oxidation products.

If the first step in the dissimilation of glycine involved hydrolytic deamination, glycolic acid would be formed. To account for the oxygen utilized during the reaction glycolic acid must be either oxidized to

<sup>2</sup> Kindly supplied by Dr. H. Diehl of the Department of Chemistry.

glyoxylic acid or decarboxylated to form methyl alcohol which would be further oxidized. The latter possibility is improbable for when *Achromobacter* cells were incubated with methyl alcohol or methylamine, no oxygen uptake was observed. It has been shown also that neither methyl alcohol nor methylamine can be demonstrated in the medium following the bacterial oxidation of glycine.

If formaldehyde is an intermediate of glycine oxidation, it should form a derivative with dimedon. Nonproliferating cells which had been cold stored for 14 days were incubated with glycine and dimedon at 30°C. To one flask were added 8 ml. of a 10% cell suspension, 8 ml. of 0.03 *M* glycine, 8 ml. of 0.2 *M* phosphate buffer, pH 6.4-6.5, and 7 ml. of 0.06 *M* dimedon. To a second flask were added 10 ml. of a 10% cell suspension, 0.8 mmole of glycine, 10 ml. of the phosphate buffer, 10 ml. of 0.16 *M* dimedon, and 10 ml. of distilled water. A current of air filtered through cotton was gently passed above the surface of the contents. After 24 hr. the incubated material was dry. It was extracted with small portions of hot toluene and the suspension filtered. The crystals obtained after the toluene was evaporated melted between 170 to 190°C. The crystals were recrystallized once from methyl cellosolve and twice from hot glacial acetic acid and melted at 188-90°C.

A third flask was prepared in a similar manner and incubated with constant shaking at 30°C. Air was bubbled through sterile water to prevent desiccation. After 15 hr. the bacteria were centrifuged and the supernatant liquor acidified with HCl. A precipitate formed, the pH was adjusted to 4.5-5.1, and the material was cold-stored overnight. The precipitate was centrifuged and recrystallized from hot methyl cellosolve. One portion of needle-like crystals melted at 145°C. (apparently unreacted dimedon). Another fraction melted at 188-91°C. and this was probably formalaldimedone. A third fraction of short stubby crystals melted at 239°C. and was unidentified; the glyoxylic acid derivate of dimedon also melts at 239°C. The entire crystalline conglomerate was recrystallized by dissolving in a minimum of hot glacial acetic acid and allowing the solution to remain at 4°C. overnight. The few crystals which formed were filtered and dried in a vacuum desiccator. The melting point was 187-9°C.

The different derivatives which had been obtained were mixed with known crystals of formalaldimedone; mixed melting points of 188-90°C. were observed. The melting point of formalaldimedone is 187-9°C. (13). It is reasonable to conclude that formaldehyde is an intermediate product of the bacterial oxidation of glycine.

The consistent occurrence of traces of formic acid suggests its origin from formaldehyde and subsequent oxidation to CO<sub>2</sub> and water. The R. Q. of 1.5 indicates that the oxidation of formaldehyde is not complete for a complete oxidation yields an R. Q. of 1.33. If hydrogen peroxide is formed during the dissimilation, then the possibility exists of an enzymatic oxidation by means of hydrogen peroxide.

Cell suspensions of *Achromobacter* were incubated in manometric cups in the presence of alcohol, glycine, and alcohol + glycine. The results (Table III) show that a coupled reaction occurred during the oxidation of glycine, suggesting the production of hydrogen peroxide. The filter paper in the alkali well of the flask containing glycine + alcohol was yellow due to the polymerization of acetaldehyde, as described by Keilin and Hartree (5).

TABLE III

*The Formation of Hydrogen Peroxide*

Each flask contained 0.5 ml. of 10% *Achromobacter* cells, 0.5 ml. 0.1 M phosphate buffer, pH 6.8, 0.5 ml. ethyl alcohol and 0.5 ml. glycine, added where indicated. Final volume = 2.0 ml. Figures corrected for endogenous respiration Time = 440 min., temperature = 30.4°C.

Experiment no	Glycine concentration	Ethyl alcohol concentration	O <sub>2</sub> utilized	O <sub>2</sub> increase	Increase
	M	M	$\mu$ l	$\mu$ l	%
1	0.04	0	434		
	0	0.04	89		
	0.04	0.04	605	82	18.9
2	0.04	0	364		
	0	0.1	(+87)		
	0.04	0.1	478	114	31.4

The bacteria quantitatively and rapidly decomposed hydrogen peroxide according to the equation:  $\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \frac{1}{2}\text{O}_2$ . (0.02 mmoles of  $\text{H}_2\text{O}_2$  was decomposed within 5 min.)

Manometric data do not reveal whether the bacteria have a peroxidase system. The oxidation of formaldehyde would involve decomposition of hydrogen peroxide with the formation of "active" oxygen; this condition would be met by peroxidase. The presence of peroxidase in *Achromobacter* was determined by adding 5 ml. of a 10% suspension of cells to 65 ml. of CO<sub>2</sub>-free water, 20 ml. of a 5% solution of pyrogallol, and 20 ml. of a freshly prepared 1% solution of hydrogen peroxide. A control flask contained the same constituents but no bacteria. After incubation for 10 min. at 10°C., the reaction was stopped by the addition of 5 ml. of 2 N HCl to each flask. The oxidation of pyrogallol by the bacteria was very rapid and accompanied by precipitation of

purpurogallin, thus showing that the bacteria contained a peroxidase system. Negligible amounts of purpurogallin were formed in the control flask.

*Biological Oxidation of Formaldehyde*

Manometric investigation of the oxidation of formaldehyde by hydrogen peroxide or barium peroxide was unsuccessful because of the great rapidity of peroxide decomposition by the bacteria. To obviate this difficulty a solution of hydrogen peroxide and formaldehyde was slowly added from a dropping funnel into a 300 ml. Erlenmeyer flask containing a bacterial suspension agitated by means of a magnetic stirrer. The results presented in Table IV show that formaldehyde is enzymatically oxidized by the bacteria in the presence of hydrogen peroxide.

TABLE IV

*Oxidation of Formaldehyde by Achromobacter in the Presence of Hydrogen Peroxide*

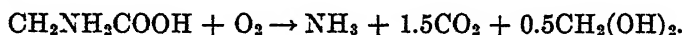
Flasks 1 and 2 contained 3.0 ml. of 0.2 *M* phosphate buffer, pH 6.8. Forty ml. of HCHO-H<sub>2</sub>O<sub>2</sub> solution was added dropwise to each flask over a period of 3.5 hr. Flask 1 contained distilled water instead of bacteria; flask 2 contained 20.0 ml. of a 7.5% bacterial suspension.

Flasks 3 and 4 contained 6.0 ml. of a 0.2 *M* phosphate buffer, pH 6.8. 100.0 ml. of HCHO-H<sub>2</sub>O<sub>2</sub> solution was added dropwise over a period of 4.5 hr. Flask 3 contained distilled water instead of cells; flask 4 contained 40.0 ml. of a 7.5% cell suspension.

Temperature = 30.4°C.

Flask no.	H <sub>2</sub> O.	HCHO		HCHO oxidized	
		Initial	Final	Enzymatically	Nonenzymatically
	<i>mmoles</i>	<i>mmoles</i>	<i>mmoles</i>	<i>mmoles</i>	<i>mmoles</i>
1	2.0	0.2	0.15	0	0.05
2	2.0	0.2	0	0.2	
3	5.0	0.5	0.47	0	0.03
4	5.0	0.5	0.05	0.45	

The over-all equation for the dissimilation of glycine may now be written:



Methylene glycol [CH<sub>2</sub>(OH)<sub>2</sub>] or hydrated formaldehyde should be identified as formaldehyde under the strongly acid conditions of the

chromotropic acid procedure. Direct addition of this reagent to deproteinized liquor obtained after the dissimilation of glycine failed to give a positive formaldehyde test. When the liquor was acidified with periodic acid and distilled, the distillate was found to contain formaldehyde. Compounds having 2 hydroxyl groups attached to adjacent carbon atoms, or a carbonyl group adjacent to a second carbonyl group or to a hydroxyl group, are oxidized by periodic acid with the liberation of formaldehyde. No relationship was found between the glycine fermented and the formaldehyde obtained by periodic acid oxidation: 0.75, 1.01, and 1.22 mmoles of glycine produced material which yielded 0.072, 0.032, and 0.058 mmole of formaldehyde, respectively. These values are far lower than would be expected if all the supposedly poly-

TABLE V  
*Derivatives Prepared from the Liquor of the  
Oxidative Dissimilation of Glycine*

Reagent	Color of derivative	Melting point of derivative °C.
2,4-Dinitrophenylhydrazine	Red-brown	148-51
p-Bromophenylhydrazine	Red-brown	107-10 <sup>a</sup> 220-5
m-Nitrophenylhydrazine	Yellow	126-8
p-Nitrophenylhydrazine	Red-brown	137-9

\* Two distinct derivatives were obtained with p-bromophenylhydrazine.

merized methylene glycol were in the fermented liquor. When deproteinized fermentation liquors were concentrated *in vacuo* (55-65°C.) and treated with Bial's orcinol reagent and thymol-sulfuric acid, pentose and carbohydrate tests were positive. The fermentation liquor failed to reduce copper in sugar reagents. Derivatives were obtained by treating the concentrated liquor with p-bromophenylhydrazine, m-nitrophenylhydrazine, and p-nitrophenylhydrazine, and were recrystallized from hot methyl cellosolve. The melting points (Table V) do not correspond with carbohydrate derivatives described in the literature.

To determine whether the bacteria assimilated the unidentified product of glycine dissimilation, cell suspensions were incubated with

glycine. At the completion of the reaction the cells were centrifuged and hydrolyzed with HCl. Aliquots of the hydrolyzate were analyzed for pentose and reducing sugars. Table VI shows that when *Achromobacter* metabolized glycine, assimilation as well as dissimilation occurs.

TABLE VI

*Analysis of Achromobacter Cells and Fermentation  
Liquor following the Dissimilation of Glycine*

Flasks 1 and 2 contained 25.0 ml. of 20% cell suspension, 10.0 ml. of 0.2 M phosphate buffer, pH 6.8 and glycine added as indicated. Final volume = 25 ml. Time = 24 hr., temperature = 30°C.

Flasks 3 and 4 contained 25.0 ml. of 14% cell suspension, 20.0 ml. of 0.2 M phosphate buffer, pH 6.8 and glycine as indicated. Final volume = 45 ml. Time = 72 hr., temperature = 30°C.

Flask no	Glycine	Fermentation liquor, pentose	Hydrolyzed cell-, carbohydrate as glucose	Carbohydrate synthesized
	<i>mmoles</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	0		48.5	
2	10	4.4	60.9	12.4
3	0		22.3	
4	15	6.3	27.9	5.6

The data indicate that the dissimilation of glycine involves the formation of hydrogen peroxide which is decomposed by peroxidase. Glycine undergoes oxidative deamination with the probable formation of glyoxylic acid which yields formaldehyde. Formaldehyde is partially oxidized to formic acid and then to CO<sub>2</sub> and water. Methylene glycol is proposed as the other product of formaldehyde, polymerizing to a nonreducing carbohydrate-like compound which reacts like a pentose. The results are summarized in Fig. 1.

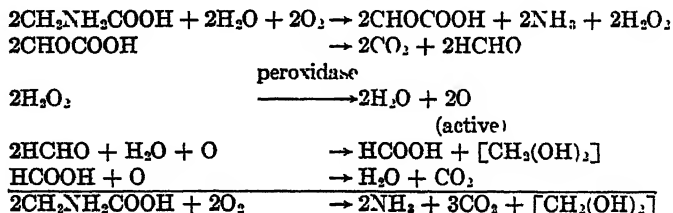


FIG. 1. Proposed scheme for the oxidative dissimilation of glycine.

## DISCUSSION

Investigations of the bacterial oxidation of glycine have pointed to either complete oxidation of the molecule or to the corresponding keto acid (14, 15, 10, 11). In neither animal nor bacterial investigations has a reaction been reported in which glycine is oxidized beyond the keto stage and yet not to completion. Whereas the formation of hydrogen peroxide is well known, and a mechanism for its formation during amino acid oxidation has been proposed (16), relatively little is known about the function of hydrogen peroxide. In the present investigations hydrogen peroxide and peroxidase are shown to be of significance in the dissimilation of glycine. The accumulation of formaldehyde, toxic in higher concentrations, is prevented by the action of hydrogen peroxide which through the intermediation of peroxidase partially oxidizes formaldehyde to formic acid. Methylene glycol is proposed as the other product of this reaction.

It has been suggested (17, 18) that methylene glycol and "active" formaldehyde polymerize to form members of the carbohydrate series, and Neuberg's experiments (19) on the polymerization of formaldehyde are well known. It is not our purpose to discuss the role of formaldehyde in photosynthesis but it is of interest to mention the glycogenic properties of glycine in connection with the formation of formaldehyde from glycine by *Achromobacter*. The data presented in this investigation are analogous to the observations of Barker and others (20, 21, 22) who found that assimilation takes place during incomplete oxidation of fatty acids and other substrates. Formaldehyde has been isolated and demonstrated as an intermediate in the metabolism of glycine. This is in agreement with the concept that "biologically active" formaldehyde, or methylene glycol, is of fundamental importance in the synthesis of carbohydrate. This proposal is strengthened by the presence of carbohydrate-like material in the medium and an increase in the carbohydrate content of the cells after the dissimilation of glycine.

## SUMMARY

1. *Achromobacter* sp. oxidizes glycine with the liberation of one mole of ammonia/mole of glycine metabolized. The R. Q. of the reaction is 1.5.
2. Hydrogen peroxide is formed during the oxidation.

3. Formaldehyde is an intermediate in the reaction and undergoes a partial oxidation or dismutation with the formation of formic acid and presumably methylene glycol. Formic acid is oxidized to  $\text{CO}_2$  and water and the proposed methylene glycol is assimilated, forming carbohydrate-like material. The decomposition of hydrogen peroxide by peroxidase is an important step in these reactions.

4. The enzyme systems involved are relatively stable; bacteria kept in the icebox for 14 days maintained full activity on glycine.

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# The D-Amino Acid Oxidase of Molds <sup>1</sup>

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## INTRODUCTION

The presence of a D-amino acid in penicillin adds to the lengthening list of places in nature where the unnatural amino acids are found. Since the absence of D-amino acids usually is thought to be the result of D-amino acid oxidase, it was believed that a study of this enzyme in penicillin producing molds was warranted. Horowitz (1944) has made a rather detailed investigation of D-amino acid oxidase in *Neurospora*, and Knight (1948) has investigated the L-amino acid oxidase of molds. The occurrence of D-amino acid oxidase in a number of molds was investigated and the properties of the enzyme in *Penicillium chrysogenum* Q176 were studied.

## EXPERIMENTAL METHODS

The molds were grown in shaken-flask culture according to the method that has been described by Koffler, Emerson, Perlman, and Burris (1945). The medium consisted of 2% corn-steep solids and 2% crude lactose at pH 4.5. A synthetic medium [Jarvis and Johnson (1947)] was used to grow the mold with different organic nitrogen sources. This was accomplished by omitting all of the ammonium nitrogen and substituting peptone or amino acids. Mold cultures were 2-3 days old at the time of harvest because the maximum quantity of enzyme was present at that time.

In earlier experiments, attempts were made to demonstrate oxidation of D-amino acids by whole pellets of mycelium or pellets blended in a Waring Blendor. Both the whole pellets and the blended pellets of mycelium had a relatively high rate of endogenous respiration so that the increase with added substrates (amino acids) was hardly measurable. Therefore, this technique was not practicable. Unless otherwise stated, a modification of the procedure of Horowitz (1944) was followed. The mycelium was removed from the medium by filtration through cheesecloth and washed with

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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distilled water, then pressed by hand through cloth to remove the excess water. The tissue contained 75–80% moisture at this stage. The pressed mycelium was ground in a mortar with sand and 2 ml. of water/g. of wet tissue. The resulting material was centrifuged for 25 min. at 3000 r.p.m.; the supernatant was removed and centrifuged for 5 min. more and then diluted with 0.25 volume of 0.1 *M* pyrophosphate buffer. The preparation usually had a pH of 8.5; sometimes an adjustment to this pH was necessary. This procedure yielded enzyme preparations that oxidized D-amino acids but had no measurable effect on L-amino acids.

Oxygen uptake was measured at 35°C. and pH 8.5 on the Warburg respirometer. Each flask contained 2.5 ml. of the enzyme preparation in the reaction chamber, 0.5 ml. of 0.1 *M* DL-amino acids or 0.05 *M* D- or L-acids in the side arm and 0.2 ml. of 20% KOH in the center well. Amino acids were tipped into the reaction chamber after 15–20 min. equilibration. Oxygen consumption was measured for a period of 30–60 min. The preparations could be held several hours in the refrigerator without loss of activity but overnight storage was not possible. There was no endogenous respiration.

Ammonia was determined by cold alkaline aeration followed by Nesslerization. Direct Nesslerization of the solutions from the reaction flasks was not possible because of substances that interfered with color development.

Pyruvic acid was determined by the method of Friedemann and Haugen (1943). DL-Alanine was deaminated in Warburg flasks; the reaction was stopped with 1 ml. of 10% trichloroacetic acid, and the flask contents were centrifuged. The supernatant was used for the pyruvic acid determinations.

## EXPERIMENTAL RESULTS

### *Properties of the Enzyme*

A number of studies were made on the properties of the enzyme. All of the enzyme preparations for these studies were made from the penicillin producing mold, *Penicillium chrysogenum* Q176.

*pH Optimum.* The effect of pH on the rate of oxygen uptake was determined for two different amino acids over a pH range of 5.5–9.5. Phosphate buffer was used from pH 5.5–8.0 and pyrophosphate buffer was used above pH 8.0. Figure 1 illustrates the effect of pH on oxygen uptake in the presence of two amino acids. In all instances the pH optimum was about 8.5.

*Temperature Optimum.* Figure 2 shows the effect of temperature on the D-amino acid oxidase with DL-methionine as substrate. The temperature optimum appeared to be above 50°C. since the rate of oxygen consumption continued to increase with rising temperature at least to this point. The equipment would not permit the use of higher temperatures. Calculations from a number of experiments showed the “mu value” for the enzyme to be approximately 6250 calories.

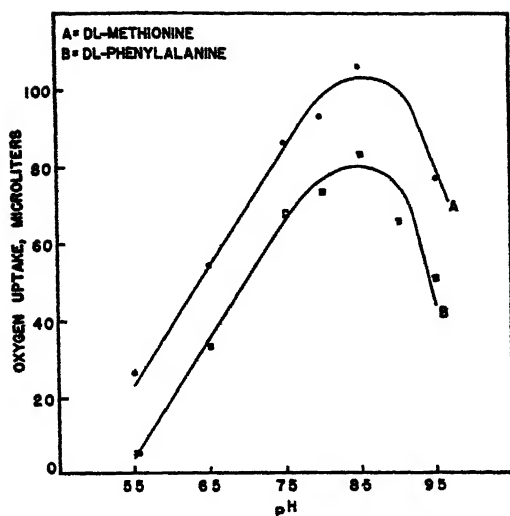


FIG. 1. The effect of pH on the activity of D-amino acid oxidase of *P. chrysogenum* Q176. The oxygen uptake of 2.5 ml. of enzyme preparation and 0.5 ml. of 0.1 M amino acid was measured for 1 hr.

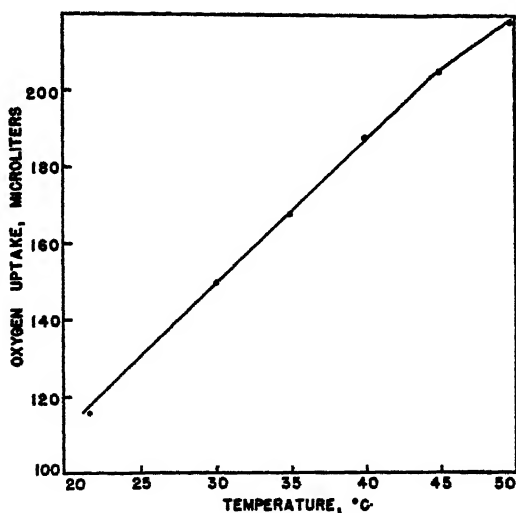


FIG. 2. The effect of temperature on the rate of oxygen uptake by D-amino acid oxidase with DL-methionine as substrate. The oxygen uptake of 2.5 ml. of enzyme preparation was measured for 1 hr.

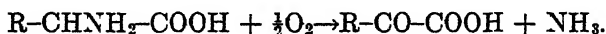
TABLE I

*The Effect of Inhibitors on D-Amino Acid Oxidase of  
P. chrysogenum Q176, DL-Methionine Substrate*

Inhibitor	Concentration M	Inhibition per cent
Iodoacetic acid	0.1	78.5
	0.01	39.4
	0.001	23.0
2,4-Dinitrophenol	0.1	63.5
	0.01	50.0
	0.001	22.0
Sodium cyanide	0.1	65.0
	0.01	0
Sodium fluoride	0.1	0
Sodium azide	0.1	0
Copper sulfate	0.1	71.0
Capryl alcohol	Saturated	80.0

*The Effect of Inhibitors.* The effect of inhibitors on the rate of amino acid oxidation is presented in Table I. With the exception of copper sulfate, capryl alcohol, iodoacetic acid, and 2,4-dinitrophenol, the inhibitors had no measurable influence on the enzyme.

*Stoichiometric Relations.* Ammonia determinations showed that 1 mole of ammonia was produced from D-alanine or DL-methionine for each 0.5 mole of oxygen that was consumed. Pyruvic acid determinations showed that one mole of pyruvic acid was formed from D-alanine for each half mole of oxygen that was consumed. No oxidation of pyruvic acid or lactic acid by the preparation could be detected. From these data (Table II) it appears that the equation for the over-all reaction is:



This is in agreement with the work of Krebs (1935).

*Action on Different Amino Acids.* All of the enzyme preparations oxidized D-amino acids; none of the preparations oxidized L-amino acids. The method of preparation either destroyed or did not extract the L-amino oxidase, since Knight (1948) has demonstrated the presence of such an enzyme in this mold.

Table III presents the order of activity on the different amino acids. Since considerable variation was encountered in the activity of different enzyme preparations, a number of experiments were run and the

TABLE II

*The Relationship of Oxygen to Ammonia and Pyruvic Acid Formation from Amino Acids by D-Amino Acid Oxidase*

Oxygen uptake $\mu$ moles	Ammonia formed <sup>a</sup> $\mu$ moles	Pyruvic acid formed <sup>b</sup> $\mu$ moles
6.6	12.4	—
6.5	—	13.0

<sup>a</sup> DL-Methionine substrate.

<sup>b</sup> DL-Alanine substrate.

figures reported represent averages; DL-methionine was used as a control in all experiments. It should be pointed out that although the preparations varied in activity, the relationship among the different amino acids remained constant. Although a number of amino acids were oxidized by the enzyme preparations, it is interesting to note the effect of various substituted groups on oxidation: for example, the substitution of a hydroxy group on the beta-carbon (serine *vs.* alanine and

TABLE III

*The Oxidation of Amino Acids by D-Amino Acid Oxidase of P. chrysogenum Q176*

Each flask contained 2.5 ml. of enzyme preparation; 0.5 ml. of 0.1 *M* DL-amino acid or 0.05 *M* D-amino acid in the side arm; and 0.2 ml. 20% KOH in the center well.

Substrate	Oxygen uptake $mm.^3/hr.$
DL-Leucine	208
D-Methionine	188
DL-Methionine	188
D-Alanine	156
DL-Alanine	146
DL- $\alpha$ -Aminobutyric acid	134
DL-Norleucine	128
DL-Phenylalanine	126
DL-Valine	112
DL-Isoleucine	64
DL- $\alpha$ -Aminocaprylic acid	50
DL-Tryptophan	38
DL-Lysine	26
DL-Threonine	14
DL-Glutamic acid	12
DL-Aspartic acid	0
DL-Serine	0

threonine *vs.*  $\alpha$ -amino butyric acid) suppressed oxidation to a marked degree. Introduction of a second amino group (lysine *vs.* norleucine) sharply decreased vulnerability of the amino acid to oxidation. There was virtually no oxidation of the dicarboxylic amino acids, glutamic and aspartic. The presence of a phenyl group (phenylalanine) had a comparatively small effect on the rate of oxidation, but the indole group (tryptophan) strongly reduced oxygen uptake. Leucine appeared to be the most available substrate of those tested, while methionine was a close second.

*Influence of the Growth Medium on D-Amino Acid Oxidase Activity*

The mold was grown on corn-steep lactose medium and on synthetic medium which contained ammonium nitrate as the sole nitrogen source; the enzyme activity from these molds was compared on several amino acid substrates. The results (Table IV) show that the mold grown on the inorganic nitrogen produced little enzyme compared to that of the mold grown on the corn-steep nitrogen. The addition of increasing amounts of ammonium sulfate to the corn-steep medium caused a progressive decrease in enzyme activity proportional to the amount of ammonium salt that was added. When the mold was grown on Difco peptone as sole nitrogen source, an active enzyme was produced. Addition of equivalent amounts of ammonium nitrogen to this growth medium reduced the enzyme activity to the level of the complete inorganic medium controls. The media were so adjusted that the pH at the time of harvest was comparable.

TABLE IV

*The Effect of the Growth Medium on the D-Amino Acid Oxidase of Penicillium chrysogenum Q176*

Each flask contained 2.5 ml. of enzyme preparation; 0.5 ml. of 0.1 M DL-amino acid in the side arm; and 0.2 ml. 20% KOH in the center well.

Growth medium	Amino acid substrate	Oxygen uptake mm. <sup>3</sup> /hr.
Corn steep-lactose	DL-Methionine	165
	DL-Leucine	168
Corn steep-lactose +1.0% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	DL-Methionine	96
	DL-Leucine	106
Synthetic	DL-Methionine	4
	DL-Leucine	5

Different substances were included in the synthetic medium to see if they would influence the enzyme content of the mycelium. The addition of calcium pantothenate, yeast extract, corn-steep ash, adenine, and riboflavine showed no influence on the amount of the enzyme in the mycelium.

It was possible to grow the mold on the synthetic medium with D- and DL-amino acids as sole nitrogen sources. The different amino acids were added to the medium in amounts to give a nitrogen content of 50 mg./100 ml. of medium. The preparations from the molds that were grown on these amino acids were active when tested on DL-methionine as substrate. The amino acids that were used as sole nitrogen sources

TABLE V

*The Effect of Amino Acids as Sole Nitrogen Source on Growth and on Ability to Oxidize the Respective D-Isomer*

Each flask contained 2.5 ml. of enzyme preparation; 0.5 ml. of 0.1 M DL-amino acid or 0.05 D-amino acid in the side arm; and 0.2 ml. 20% KOH in the center well.

Amino acid	Dry weight of mycelium mg./100 ml	Oxygen uptake mm. <sup>3</sup> /hr.
DL-Glutamic acid	587.9	12
D-Alanine	553.1	156
D-Methionine	340.8	188
L-Methionine	336.8	192
DL-Phenylalanine	277.6	126
L-Phenylalanine	256.2	131
DL-Serine	247.0	0
DL-Tryptophan	222.7	38
DL- $\alpha$ -Aminoisobutyric acid	73.9	0

for growth were compared as to their ability to serve as oxidizable substrates for the enzyme (Table V). The best growth occurred on glutamic acid, although this amino acid was oxidized poorly by the enzyme. Likewise serine and tryptophan supported good mycelial growth but were deaminated slowly or not at all.

Methylene blue could serve as the hydrogen acceptor for the deaminase in Thunberg tubes, but the reaction was sluggish. Knight (1948) found similar results for the L-amino acid oxidase of this mold.

All attempts to concentrate and purify the enzyme, from fresh mycelia and from stable acetone-dried mycelia were unsuccessful.

*D-Amino Acid Oxidase of Various Molds*

The D-amino acid oxidase content of a number of molds was determined; both penicillin producers and those that do not produce penicillin were studied. In making these studies, enzyme preparations of each mold were tested on a representative group of amino acids. In order to make the results comparable, moisture was determined on the mold mycelia and the oxygen uptakes expressed on the basis of dry weight. The data for these experiments are found in Table VI. It should be pointed out that none of the enzyme preparations oxidized the L-amino acids. It was generally possible to obtain enzyme preparations

TABLE VI

*The D-Amino Acid Oxidase Activity of Penicillin and Non-Penicillin-Producing Molds*

Each flask contained 2.5 ml. of enzyme preparation; 0.5 ml. of 0.1 *M* DL-methionine substrate; and 0.2 ml. 20% KOH in the center well.

Mold	Oxygen uptake mm. <sup>3</sup> /hr./mg.
<i>P. chrysogenum</i> Q176	188
<i>P. chrysogenum</i> X1612	97
<i>P. notatum</i> NRRL1951-B25	85
<i>P. notatum</i> 832	76
<i>P. notatum</i> 174	37
<i>P. sanguineum</i>	2
<i>P. roqueforti</i>	296
<i>P. roqueforti</i> nutant	266
<i>Aspergillus niger</i>	96

capable of oxidizing D-amino acids from all of the molds that were tested. The best penicillin-producing mold, *P. chrysogenum* Q176, yielded enzyme preparations of considerably greater activity than other penicillin producers. However, the most powerful D-amino acid oxidase extracts were obtained from *Penicillium roqueforti*, which is not a penicillin producer. Leucine usually was the most rapidly oxidized substrate among those tested; however, *Aspergillus niger* preparations oxidized methionine at a more rapid rate than any other substrate. *Penicillium sanguineum* virtually was incapable of oxidizing D-amino acids; likewise, it has a low L-amino acid oxidase content (Knight, 1948).

## DISCUSSION

Although no attempt was made to put the D-amino acid content of the various molds on a strictly quantitative basis, it is evident that the enzyme was present in all of the molds. In many respects the properties of the D-amino acid oxidase from *P. chrysogenum* Q176 were similar to the properties of the L-amino acid oxidase (Knight, 1948) except, of course, the specificity for the respective isomer. Except for the method of preparing the tissue, the properties of the crude preparations and the conditions under which the enzymes were produced seem to be identical. A study of the chemical and physical make-up of the two enzymes was not in the realm of this work.

Although the D-amino acid content of mycelium was low during the period of penicillin formation it does not seem likely that the low oxidase content and the formation of a D-amino acid complex (penicillin) are directly related. The fact that the addition of D- and DL-amino acids, including penicillamine, to penicillin fermentations at various times never has increased penicillin production (unpublished data) seems to indicate that penicillin formation is not merely a mechanism for detoxifying a nonphysiological amino acid.

It is noteworthy that *P. chrysogenum* Q176 could grow well on any of a number of DL-amino acids, D-methionine, or D-alanine as the sole nitrogen source and that there was no apparent relationship between the ability to deaminate the amino acid and the ability to use the acid as a nitrogen source. It is peculiar, also, that the least D-amino acid oxidase was produced with ammonia as the nitrogen source. If the organism synthesizes both amino acid isomers and then deaminates the D-form, as theory provides, the most oxidase would be expected where the most synthetic activity occurs, i.e., with ammonia as the nitrogen source and not with corn-steep liquor, peptone, or amino acids. Such is not the case with *P. chrysogenum* Q176. The role of D-amino acid oxidase in the mold, as in other tissues, is not evident.

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## SUMMARY

Cell-free preparations from penicillin-producing molds, other *Penicillia* and *Aspergillus niger* brought about the oxidation of D-amino

acids with the liberation of one mole of ammonia and one mole of alpha-keto-acid for each mole of D-amino acid oxidized and for each one-half mole of oxygen consumed. Except for isomer specificity and the method of preparation, the crude D- and L-amino acid oxidases are very similar. The function of the amino acid oxidase is not apparent.

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# Mechanism of the Lipotropic Action of Estrogen

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## INTRODUCTION

In two previous studies (1, 2) it has been shown that estrogens, given orally, exert a lipotropic effect in rats maintained on a diet deficient in lipotropic factors. In particular, estrogens allow more efficient use of methionine as a lipotropic agent. During the course of this work it was observed, in a preliminary trial, that rats with estrone pellets implanted in the spleen did not experience a similar protective effect. The presence of estrone in the spleen does not induce estrus in the rat because the hormone is inactivated by the liver before it can reach the systemic circulation. The oral administration of estrogens is likewise inefficient, because in this case also, at least a major portion of the hormone is carried directly to the liver by the portal vein and there inactivated. However, the animals in which a lipotropic effect of estrogen had been demonstrated were constantly in estrus. In this instance there was, therefore, a systemic action from the estrogen which survived the hepatic passage. Since, in the pellet experiments, the animals were in anestrus, the possibility could be entertained that the lipotropic manifestations of estrogen depend on systemic action rather than on a local hepatic effect. The superiority of ethinyl estradiol over the other estrogens in lipotropic activity (2) is in keeping with this explanation. Although the relative potencies of oral and parenteral administration of ethinyl estradiol in relation to other estrogenic hormones have not been precisely determined, the compound is known to produce pro-

found systemic effects when given orally in very small doses. Data pertinent to this problem have been sought by comparison of the effect of estrone pellets implanted subcutaneously, which will allow maximum systemic effect of the hormone, with pellets placed in the spleen.

## METHODS

Young adult female rats were ovariectomized. After 2 weeks, pellets of estrone (in one experiment, mixed estrogen from pregnant mares' urine) weighing approximately 5 mg. were implanted in the spleen or subcutaneously in the back. Control animals were ovariectomized and an operation simulating implantation carried out at the time the pellets were implanted in the test animals. The animals were given the stock ration for about 2 weeks after the operation to allow complete recovery.

During the experimental period the rats received an alipotropic diet or the same diet plus a supplement of 50 mg. of methionine daily. The diet, the general experimental procedure, and the method of analysis for liver fat have been described in the earlier papers (1, 2).

Vaginal smears were examined repeatedly throughout the experiments to determine the presence or absence of estrus. Pellets were weighed at the beginning and end of the experiment so that the amounts of hormone absorbed daily could be compared.

These experiments were carried out over an extended period of time and in two laboratories, Cleveland and Philadelphia, under, in so far as possible, identical conditions. Control groups were run with each experiment since there may be considerable variation in the level of liver fat found in experiments carried out at different times. The values found for the treated groups must be compared only with that of the control group which was run simultaneously.

## RESULTS

The lack of any protective effect of estrone after intrasplenic implantation was quite evident in the first experiment (Table I). The fat content of the liver was the same in the control and experimental groups. In view of the fact, however, that estrone without the aid of methionine may not always exert a significant effect this result is not conclusive.

In the second experiment animals receiving methionine were compared with those without methionine. Most of the animals with splenic pellets showed the typical picture of complete absence of estrus. Half of these animals were given the methionine supplement. Six animals exhibited continuous estrus after the pellet was planted, due, no doubt, to adhesions between the spleen and the abdominal wall which allowed direct access of the hormone to the systemic circulation. These 6

TABLE I  
Effect of Route of Administration of Estrogen on Its Lipotropic Activity

Treatment	No. of rats	Liver		Rat weight		Food intake (g./day)	Estro-ne absorption (ug./day)	Remarks
		Weight (g.)	Total fat (%)	Initial (g.)	Change (%)			
Experiment 1								
No treatment	10	8.6 ± 0.73	28.7 ± 2.6 <sup>a</sup>	159 (119-190)	- 7.0 ± 2.0	6.6 ± 0.40		None in estrus
Estro-ne pellet in spleen	12	7.9 ± 0.62	27.8 ± 2.8	160 (130-180)	- 5.2 ± 0.71	6.5 ± 0.34		Two rats in estrus
Experiment 2								
No treatment	11	6.5 ± 0.26	12.9 ± 1.7	180 (160-202)	- 11.5 ± 0.90	6.5 ± 0.23		
Estro-ne pellet in spleen	11	6.0 ± 0.23	10.3 ± 1.3	165 (151-195)	- 9.6 ± 0.65	6.2 ± 0.16		None in estrus
Meth., 50 mg./day and Estro-ne pellet in spleen	11	6.6 ± 0.31	8.6 ± 0.51	178 (145-184)	- 5.4 ± 2.0	6.3 ± 0.29		None in estrus
Meth., 50 mg./day and Estro-ne pellet in spleen	10	6.0 ± 0.23	9.2 ± 1.0	159 (123-203)	- 5.5 ± 1.7	6.0 ± 0.37		None in estrus
Meth., 50 mg./day and Estro-ne pellet in spleen	6	6.3 ± 0.36	0.2 ± 0.28	145 (122-158)	- 4.4 ± 2.8	6.0 ± 0.29		All in estrus
Experiment 3								
No treatment	10	14.0	15.4 ± 1.47	246 (215-337)	- 3.1 ± 1.8	9.7 ± 0.8		
Estro-ne pellet in spleen	10	12.6	13.0 ± 2.6	241 (192-292)	- 3.7 ± 2.0	9.0 ± 0.5		None in estrus
Estro-ne pellet subcutaneous	10	13.1	7.8 ± 0.49	254 (240-341)	- 0.1 ± 1.9	9.2 ± 0.5	12.2 ± 1.0 16.2 ± 1.7	All in estrus
Experiment 4								
No treatment	10	12.0 ± 0.6	12.8 ± 1.3	261 (231-294)	- 0.2 ± 2.5	8.7 ± 0.5		
Estro-ne pellet in spleen	10	11.8 ± 0.5	10.2 ± 1.7	260 (240-272)	- 2.0 ± 2.2	8.5 ± 0.4		None in estrus
Estro-ne pellet subcutaneous	10	10.5 ± 0.4	6.6 ± 0.5	234 (208-257)	+ 1.1 ± 2.8	8.1 ± 0.4	11.9 ± 3.2 20.4 ± 3.3	All in estrus
Experiment 5								
Meth., 50 mg./day	11	6.9 ± 0.9	13.3 ± 1.3	256 (190-300)	- 8.5 ± 2.0	6.4 ± 0.3		None in estrus
Meth., 50 mg./day and Estro-ne pellet in spleen	13	7.6 ± 0.2	11.0 ± 0.8	242 (212-277)	- 1.7 ± 1.3	7.1 ± 0.3	7.5 ± 1.0	All in estrus
Meth., 50 mg./day and Estro-ne pellet subcut.	11	7.1 ± 0.2	7.3 ± 0.3	232 (216-272)	- 2.5 ± 1.6	7.5 ± 0.2	9.0 ± 2.3	All in estrus

<sup>a</sup> Standard error of the mean.

<sup>b</sup> Natural estrogene substance—mixed estrogen from pregnant mares' urine.

animals were given methionine and treated as a separate group. The liver fat of the control animals was much lower than that found in the preceding experiment. The value in the group with splenic pellets was slightly lower than in the control group but, in confirmation of the first study, the difference was not significant. Methionine, given alone without estrogen, resulted, as would be expected, in a moderate decrease of liver fat from the 12.9% of the controls to 8.6%. The presence of the splenic pellet in the group without estrus caused no further reduction of liver fat: in fact, the average value of 9.2% was slightly higher than the value found with methionine alone. In the group which showed constant estrus, however, the liver fat was 6.2%, a value significantly lower than that of either of the other two groups ( $p < 0.01$ ). Thus the lipotropic response to estrogen is correlated with the appearance of estrus, which, in turn, is a reflection of the systemic effect of the hormone beyond any direct influence on the liver.

A more specific comparison of the effect of estrogen introduced into the systemic circulation and presented directly to the liver was possible by the use of subcutaneous pellets. In 2 experiments, the lipotropic effect of pellets alone was studied; in a third, methionine was given as a supplement. In no case was there any significant effect from the splenic implant. In all 3 experiments the presence of the pellet in the subcutaneous tissue resulted in almost 50% reduction of the liver fat, a highly significant degree of protection.

In these experiments there was considerable variation in the amount of estrone absorbed from the splenic and subcutaneous implants. In Expts. 3 and 5 the amounts absorbed by the two routes may be considered essentially identical as far as potential biological effects are concerned. In Expt. 4, however, significantly more of the hormone was absorbed from the subcutaneous pellet, 20.4  $\mu\text{g.}/\text{day}$  as compared with 11.9  $\mu\text{g.}$  With the pellet in the spleen, however, the liver received the full benefit of all of the hormone, while in the case of subcutaneous implantation the concentration in hepatic blood, and probably the total amount of intact hormone ultimately delivered to the liver, was considerably reduced. Nevertheless, significant protection against fatty liver was observed only in the latter instance where it may be assumed that the amount of hormone delivered to the liver was small in comparison with that yielded to the systemic circulation.

## DISCUSSION

The results of the present experiments, with the previous findings, are consistent with the conclusion that estrogen, when delivered directly to the liver, either orally or from a splenic implant, does not exert a lipotropic effect unless a certain component of overflow reaches the systemic circulation.

When this series of experiments was begun, the tentative hypothesis was that estrone exerted a direct lipotropic effect on the liver. The work was initiated by the observation, during the course of experiments utilizing estrone pellets implanted in the spleens of castrated female rats as a means of assay of nutritional hepatic injury of the cirrhotic type (3, 4), that incidence and degree of cirrhosis were less than usually seen, an effect which might be attributed to the constant drainage of estrone into the liver. A systemic effect was not particularly considered since the animals showed no estrus for many weeks. However, leakage of estrone, as evidenced by the appearance of estrus, began some time before severe cirrhosis had developed, and slight escape at a level too low to cause estrus may have begun somewhat earlier. Thus the protective effect of estrone in this case may have been exerted after mild liver damage allowed estrone to enter the circulation.

If estrogen exerts its lipotropic effect, not by direct action on the liver cells, but through some indirect mechanism which is brought into play when it reaches the systemic circulation, suspicion may logically be directed toward the pituitary gland. It is well known that estrogen inhibits gonadotropin (F. S. H.) production by this gland, and there is indirect evidence that the secretion of other hormones such as prolactin and growth hormone may likewise be reduced (5, 6). The pituitary principle which promotes fat storage in the liver (7) might be similarly inhibited by the administration of estrogen.

A pertinent objection to a theory of pituitary mediation might be made on the grounds that the fatty liver produced by pituitary extract appears to depend upon an increased transfer of body fat to the liver, while the hepatic fat which appears under the influence of an alipotropic diet accumulates in the liver because of an impairment in the release of fatty acid from this organ (8, 9). However, although the mechanisms are different, it is a reasonable assumption that a decreased supply of fat to the liver would counteract the tendency of the diet to cause excessive storage through its suppression of hepatic out-

put. There is also evidence that the pituitary is involved directly in the internal control of hepatic fat metabolism. Acetone body production by liver tissue *in vitro* is enhanced by the addition of pituitary extract to the incubating medium (10).

If a pituitary factor is important for the attainment of full effects from an alipotropic diet, hypophysectomy should inhibit the development of a fatty liver in rats fed such a diet. There is a suggestion from previous experiments that this is the case (11). However, hypophysectomized animals tolerate the diet badly, and their very poor physical condition combined with a marked weight loss makes the interpretation of results difficult. Hypophysectomy per se does not influence the fat content of the liver of dogs fed a normal meat diet not restricted in lipotropic factors (12).

An alternative to the theory of pituitary mediation is the release or conservation through the action of estrogen, of lipotropic material arising from extrahepatic sources. Such an activity could hardly be confined to a simple anabolic conservation of protein, since testosterone, a more potent hormone than estrogen in this respect, has not been found to exhibit lipotropic activity (2).

### SUMMARY

Estrone, implanted subcutaneously and absorbed through the systemic circulation had definite lipotropic effect in castrate female rats. When the pellet of estrone was placed in the spleen and drained directly into the liver there was no protection against the development of fatty liver. In a few cases in which the appearance of estrus in rats with splenic pellets indicated that a portion of the hormone was escaping inactivation in the liver, the liver fat was reduced.

The lipotropic effect of estrone appears to depend upon an extrahepatic mechanism rather than upon direct hepatic action. The extrahepatic effect might be mediated through the anterior pituitary gland.

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# Investigations on Respiration, Growth, and Fat-Production of *Rhodotorula gracilis* When Cultivated in Media Containing Different Carbohydrates

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## INTRODUCTION

Several types of yeast may form fat in large amounts. Enebo, Elander, Berg, Lundin, Nilsson, and Myrbäck (1) and Enebo, Anderson, and Lundin (2) have found that this is particularly true of certain species of *Rhodotorula*. Particularly striking was the fat forming capacity of *Rhodotorula gracilis* Rennerfelt, which was found to form fat up to an amount of 63% of its dry weight.

For these investigations of fat formation by *Rhodotorula gracilis*, incubations were made in media which contained invert sugar as a carbohydrate source. As industrial utilization of this fat-forming capacity seems possible, it was of interest to know whether fat may be formed in media containing other saccharides.

Certain acid hydrolyzates of wood form a particularly cheap source of sugar-containing material which consists mainly of mannose and pentoses.

The growth of *Rhodotorula gracilis* and the subsequent fat formation with these saccharides as substrates was therefore investigated and a general investigation was made of the capacity of *Rh. gracilis* to utilize some mono- and disaccharides.

In the majority of experiments the value of a saccharide as a nutrient was investigated by incubation and subsequent measurement of respiration of the yeast in a medium containing the saccharide—a very rapid method. Alternatively the growth of the yeast was measured when incubated with media containing different saccharides. In addition, measurements were made of the fat formation taking place upon incubation in media containing xylose.

## EXPERIMENTAL

1. *Mono- and Disaccharides*

The Warburg method was used for these experiments.

Yeast well washed with 0.05 *M*  $\text{KH}_2\text{PO}_4$  solution was added to 0.05 *M*  $\text{KH}_2\text{PO}_4$  solution to give a 0.5% suspension (calculated as dry weight). Two ml. of this solution was placed in the main Warburg chamber. The yeast used had been cultivated on beer wort (40% beer wort and 2.5% agar) and was 7–12 days old. The side-bulb of the Warburg contained 0.5 ml. of a 5% solution of the saccharide to be investigated. All measurements of respiration were made at 25°C.

At first the respiration was measured every half hour without the addition of sugar. This respiration, which takes place at the cost of the nutrient reserves in the yeast, was weak and remained constant for

TABLE I

*Rate of Respiration of Rhodotorula gracilis in Media of  
Different Mono- and Disaccharides*

Glucose	100
Fructose	102
Mannose	108
Galactose	64
Saccharose	72
Maltose	80
Lactose	0
Arabinose	21
Xylose	29

several hours. After 1.5 hr. the sugar solution was tipped from the side chamber and respiration was recorded after the same intervals. The addition of a saccharide caused an increase in respiration which varied according to the ability of the yeast to utilize the saccharide for its respiration processes. The nutrient value of a saccharide was calculated from the difference between respiration rates before and after addition of the saccharide. The first readings after addition of sugar solutions were ignored; later readings were constant over several hours.

The relative rates of respiration (glucose = 100) for different saccharides are given in Table I.

The table shows that glucose, fructose, and mannose are of approximately equal value as respiratory substrates for *Rhodotorula gracilis*.

The rate of respiration is somewhat weaker with galactose, saccharose, and maltose as substrates and it is very weak in media containing one of the pentoses, arabinose or xylose. Lactose was not consumed at all in these experiments. In the next section it will be shown how *Rh. gracilis* may become adapted to utilize xylose.

## 2. Adaptation of *Rh. gracilis* to the Consumption of Xylose

Experiments were made to see if by cultivation in a xylose-containing medium *Rh. gracilis* could become adapted to utilize this material for growth and respiration.

TABLE II  
*Adaptation of Rhodotorula gracilis to Xylose*  
Respiration in glucose medium = 100  
Relative values for respiration on xylose

Time of cultivation, days	Yeast cultivated in glucose medium	Yeast cultivated in xylose medium
Experiment A		
2	30	32
4	25	24
5	29	32
7	26	35
9	28	73
11	25	76
Experiment B		
3	32	30
5	27	26
7	25	32
8	27	45
9	29	68
10	24	72
11	27	69

Two media were used in parallel for the cultivation of the yeast, one with glucose and the other with xylose. They had the following composition: sugar, 40 g.;  $(\text{NH}_4)_2\text{SO}_4$ , 15 g.;  $\text{KH}_2\text{PO}_4$ , 4.7 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 3 g.;  $\text{NaCl}$ , 1.5 g.;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.5 g.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.015 g., made up to 1000 ml. The yeast was cultivated at 25°C.

The respiration of the yeast was studied simultaneously in glucose- and xylose-containing media. The Warburg method was used. Four experiments were performed. The results obtained in two of these are given in Table II.

Table II shows that in a medium containing xylose, yeast not adapted to xylose respired at a rate which was only 24–32% of the respiration in a glucose medium (cf. Table I); after cultivation for 7–8 days in a medium containing xylose, adaptation of the yeast to xylose took place, and after 9 days the maximum adaptation was obtained, the respiration on xylose being then 68–76% of the respiration on glucose. From Table III it may be seen that the growth in a medium containing xylose begins to be considerable after about 9 days.

*3. Growth of Rh. gracilis in Media Containing Glucose and Xylose, Respectively*

In these experiments the solutions used were prepared as in Sec. 2. The yeast was cultivated in 200-ml. Erlenmeyer flasks containing 100 ml. of the medium. The flasks were inoculated with yeast cultivated on beer wort agar, to each flask an amount of yeast corresponding to 6 mg. dry weight being added. Agitation, which appeared to promote

TABLE III  
*Cultivation Experiments with Rhodotorula gracilis in Media Containing Glucose and Xylose*

Cultivation time, days	Sugar taken up by yeast, g./100 ml.	Yeast formed, g./100 ml.	Economic coefficient
<i>a. Medium containing glucose</i>			
2	0.67	0.391	58.4
3	2.17	0.880	40.6
5	3.51	1.182	33.7
7	4.01	1.280	31.9
9	4.05	1.258	31.1
<i>b. Medium containing xylose</i>			
2	0.10	0.087	87.0
3	0.21	0.066	31.4
5	0.25	0.097	38.8
7	0.95	0.322	33.9
9	2.17	0.658	30.3
12	4.12	1.150	27.9

growth, was carried out mechanically during the course of the experiment. The temperature was maintained at 26°C.

After successive intervals of time the quantity of yeast formed and the sugar content of the solution were both determined.

The yeast was washed and separated by centrifugation and dried to constant weight at 105°C. The sugar content was determined according to Schoorl (3) as modified by the analytical committee of the Cellulose Industry's Central Laboratory at Stockholm (4).

Two such experiments were performed with similar results. Table III shows the result of one.

TABLE IV

*Cultivation Experiments with Rhodotorula gracilis, Adapted to Xylose, in Media Containing Glucose and Xylose*

Cultivation time, days	Sugar taken up by yeast, g./100 ml.	Yeast formed, g./100 ml.	Economic coefficient
a. Medium containing glucose			
2	0.72	0.368	51.1
3	1.88	0.790	42.0
5	3.22	1.204	37.4
7	3.97	1.298	32.7
b. Medium containing xylose			
2	0.69	0.331	48.0
3	1.71	0.698	40.8
5	3.16	1.156	36.6
7	3.99	1.274	31.9

It is evident from Table III that initially the growth was stronger in the medium with glucose than in that containing xylose. After 5 days the growth in the glucose substrate had already reached its maximum value but the growth of yeast in the xylose substrate was still low. It is clear that 5 days are required for the yeast to adapt itself to the assimilation of xylose. The amounts of yeast at the conclusion of the experiment were almost equal in both media.

The economic coefficient (grams of yeast formed/100 g. of sugar used) fell considerably during the experiment. This was caused by an

autolysis gradually setting in. The yeast substance released by autolysis was not included in the determination of the "yeast weight," and the values for the amounts of yeast found are therefore somewhat lower than the actual amounts formed. This gives a lower economic coefficient than was actually the case. At the stage of fermentation when approximately half the sugar had been used, the economic coefficient for the yeast cultivated in a glucose medium was 40.6 and for that cultivated in a xylose medium, 30.3. In view of the fact that these stages of fermentation were reached after 9 and 3 days for xylose and glucose, respectively, the differences between these coefficients are not unduly large.

In a new experiment it was found that a yeast which had already been adapted to xylose was able to grow immediately, without a lag-phase, in a medium containing xylose.

It may be seen from Table IV that the growth of the yeast in the medium containing xylose, including the growth in the first stages, is almost as good as in the medium containing glucose. The economic coefficient is not essentially lower for the yeast grown in xylose medium.

In conclusion, it can be said that *Rh. gracilis* can grow well on xylose; the growth is somewhat retarded, but this delay can be avoided by first adapting the inoculation yeast to xylose.

#### 4. Fat Production After Adaptation in a Medium Containing Xylose

As stated above, fat production in *Rh. gracilis* has been demonstrated only in yeast which has been cultivated in a glucose medium. We have therefore investigated the fat production which takes place when *Rh. gracilis* is incubated in a medium containing xylose.

A medium used for the production of a *Rh. gracilis* yeast rich in fat should (2) be low in nitrogen. We used the following solution: xylose,

TABLE V  
*Fat Production of Rhodotorula gracilis in Medium Containing Xylose*

Experiment	Yeast formed, g./100 ml.	Fat, mg./g. yeast	Sugar assimilated by yeast, g./100 ml.	Economic coefficient	Fat coefficient [Rappel (6)]
A	0.661	248	2.27	29.1	7.2
B	0.832	275	2.42	34.4	9.5
C	0.720	264	2.30	31.3	8.3

40 g.; asparagine, 1 g.;  $\text{KH}_2\text{PO}_4$ , 2 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g.;  $\text{NaCl}$ , 0.5 g.;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.5 g.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.005 g., made up to 1000 ml.

The yeast was cultivated in 200-ml. flasks containing 100 ml. nutrient solution; the flasks were shaken at  $25^\circ\text{C}$ . and after 4–7 days the amount of yeast formed and its fat content were determined by Reichert's method (5). Three experiments (A, B, and C) were performed.

It is evident from the experiments that *Rh. gracilis* can form fat in nutrient solutions containing xylose as carbohydrate. The formation of fat is rather small compared with the formation in nutrient solutions containing glucose. A more complete adaptation would probably result in a higher fat forming capacity.

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#### CONCLUSION

The investigations of *Rhodotorula gracilis* here reported show that this yeast is able to utilize the saccharides occurring in wood hydrolyzates. Xylose is less suitable but, after adaptation to xylose in a medium containing xylose, *Rh. gracilis* becomes capable of utilizing this pentose also. The economic coefficient is then satisfactory. It is also shown that fat formation in *Rh. gracilis* is possible if the pentose, xylose, is used as sugar in the medium.

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# Studies on the Intermediate Metabolism of Tryptophan Labeled with Radioactive Carbon <sup>1</sup>

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## INTRODUCTION

Recent investigations have revealed that nicotinic acid is formed from tryptophan in a number of species of animals and in *Neurospora* (1, 2, 3, 4). In the series of reactions leading to nicotinic acid, the side chain of typtophan is lost in some unknown manner (1, 2). The experiments reported here were undertaken to determine the pathway of catabolism of this side chain.

Earlier work to determine whether tryptophan is glycogenic or ketogenic was inconclusive. Dakin (5) observed that L-tryptophan did not produce appreciable extra glucose in phlorizinized dogs. Borchers, Berg, and Whitman (6) were unable to decide definitely whether tryptophan was glycogenic or ketogenic in starved rats. In our experiments, it was found that the labeled beta-carbon of tryptophan appeared in the urinary glucose of phlorizinized rats. The distribution of the label in the glucose suggested the intermediary formation of a methyl-labeled acetyl derivative. However, none of the radioactive carbon appeared in the ketone bodies. Confirmatory evidence of formation of a two-carbon derivative from tryptophan was obtained from other experiments. Analysis was also carried out of the C<sup>14</sup> content of the amino acids of the proteins from a rat fed a large dose of labeled tryptophan.

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## EXPERIMENTAL

The DL-tryptophan<sup>1</sup> used in these experiments was labeled with C<sup>14</sup> on the beta-carbon and had a radioactivity of 0.85  $\mu$ c./mg. or approximately 106,000 counts min./mg. Since administration of a small amount of this highly active material might have resulted in most of it being incorporated into the body proteins, 3 mg. of it was diluted with 500 mg. of nonradioactive L-tryptophan and administered in large doses.

Fasting male rats were injected daily with 100–200 mg. phlorizin/kg. body weight. On the day following the first injection, the D:N ratio of the urine was between 2.0 and 2.9 and on subsequent days varied between 2.6 and 4.0. On the third day after the onset of phlorizin administration, the tryptophan was injected subcutaneously as an aqueous suspension. The urine was collected for 24 hr. and the animals autopsied. The administered tryptophan was completely absorbed from the site of injection.

*Glucose*

An aliquot of urine containing about 500 mg. of glucose was clarified with mercuric sulfate and barium carbonate (7). The phenylglucosazone was prepared using 4 g. of phenylhydrazine hydrochloride and 6 g. of sodium acetate/g. of glucose. The osazone was repeatedly crystallized until the correct melting point and constant specific activity were obtained. It was then converted to the phenylglucosotriazole according to the procedure of Hann and Hudson (8) by treatment with cupric sulfate. The purity of this compound was established by determinations of melting point, mixed melting point, nitrogen analysis, and constant specific activity after repeated crystallizations.

*Ketone Bodies*

Another aliquot of urine was treated with cupric sulfate and lime and the filtrate refluxed with Denige's reagent and dichromate (9). The washed mercury-acetone precipitate was redissolved in 1 *N* HCl and distilled, the distillate being collected in Denige's reagent. The mercury-acetone precipitate was again prepared and counted. The activity was extremely low (between 130 and 170 counts/min./mmole). The acetone was next obtained as the 2,4-dinitrophenylhydrazone using the apparatus of Van Slyke, MacFadyen, and Hamilton (10). The mercury-acetone precipitate dissolved in 4 *N* HCl was placed in one arm and an alcoholic solution of 2,4-dinitrophenylhydrazine in the other. The apparatus was evacuated after cooling the liquids in Dry Ice, the acetone was distilled over by immersing the flask containing mercury-acetone in hot water and then the whole apparatus was immersed in hot water. On cooling, crystals of 2,4-dinitrophenylhydrazone appeared which were purified by re-

<sup>1</sup> Synthesized and generously supplied by Drs. M. Calvin and C. Heidelberger, Radiation Laboratory, University of California [*J. Biol. Chem.* **179**, 139 (1949)].

TABLE I

*Radioactivity of Urinary Constituents of Rats Injected with Tryptophan- $\beta$ -C<sup>14</sup>*

Rat no.	Dose		Urine		Glucose			Ketone bodies		Urea counts min./mmole
	mg	Total count/min.	Total counts/min.	Per cent of dose	Total mmole	Counts/min./mmole	Per cent of dose	Total mmole	Counts/min./mmole	
1	168	124,000	62,700	50.6	4.40	6,420	22.7	0.89	0	102
2	215	195,000	100,000	51.0	3.95	6,920	13.9	2.25	0	131

crystallization (m.p. 126–128°). The compound had the correct nitrogen content. The low radioactivity of the mercuric precipitate could be removed by this procedure.

### Urea

The radioactivity of urea in the urine was determined by treating the urine, previously freed from dissolved CO<sub>2</sub>, with an extract of Jack bean meal and collecting the liberated CO<sub>2</sub> in CO<sub>2</sub>-free sodium hydroxide. The carbonate was precipitated with BaCl<sub>2</sub> and analysed as BaCO<sub>3</sub>.

### Urine

An aliquot of the urine was evaporated on an aluminum disc and counted.

The C<sup>14</sup> analyses of the products are given in Table I. An appreciable portion of the C<sup>14</sup> was found in the glucose whereas the ketone bodies were inactive. In order to determine the distribution of C<sup>14</sup> in the glucose, it was converted to the phenylglucosotriazole and degraded.

### Degradation of Phenylglucosotriazole

The oxidation of phenylglucosotriazole with NaIO<sub>4</sub> (8) yields 1 mole of an aldehyde, 2-phenyl-4-formylosotriazole, from the first three carbon atoms, 2 moles of formic acid from the fourth and fifth carbon atoms and 1 mole of formaldehyde from the sixth carbon. Most of the formylosotriazole can be filtered off from the oxidation product and the small amount in solution in the filtrate extracted with ether in a separatory funnel. It was purified by recrystallization from alcohol and water. The formaldehyde in the filtrate was precipitated with dimedon (11). The precipitate was purified by dissolving it in dilute sodium hydroxide and reprecipitating with acetic acid. The resulting methylene-bis-methone melted sharply, showed no depression when mixed with an authentic sample and contained no nitrogen. The specific activity was not changed by recrystallization. The methylene-bis-methone can be purified further by heating with glacial acetic acid (11), but this was never found to be necessary. The filtrate, after recovering the methylene-bis-methone, was extracted with ether to remove excess dimedon, acidified, and the formic acid in the solution oxidized with mercuric oxide (12) to carbon dioxide and counted as barium carbonate.<sup>4</sup>

<sup>4</sup> An essentially similar method for the separation of the oxidation products of the glucosotriazole is being used by Dr. W. Z. Hassid and co-workers (personal communication).

TABLE II

*Distribution of  $C^{14}$  in the Urinary Glucose of Phlorizinized Rats After Administration of Tryptophan- $\beta$ - $C^{14}$  or  $C^{14}H_3\cdot COOH$*

Compound	Counts/min./mmole	
	Tryptophan	Acetate
Phenylglucosotriazole (Carbons 1 to 6)	3,310	9,460
2-Phenyl-4-formylsotriazole (Carbons 1 to 3)	1,630	4,710
Formic acid (Carbons 4 and 5)	375(750) <sup>a</sup>	1,130 (2,260) <sup>a</sup>
Formaldehyde (Carbon 6)	840	2,290

<sup>a</sup> Figures in parenthesis represent the activity for 2 mmoles. One mole of glucosotriazole gives two moles of formic acid.

The distribution of the activity in the glucose is shown in Table II. To confirm the validity of the method,  $C^{14}$ -methyl labeled acetate was administered to phlorizinized rats and the urinary glucose similarly degraded. These results are also presented in Table II. The observed distribution of radioactivity in the different carbons of glucose indicates the intermediary formation of an acetyl derivative the methyl group of which comes from the beta-carbon of tryptophan. In order to confirm this suggestion, experiments on the acetylation of *p*-aminobenzoic acid were carried out.

### *Acetate and Pyruvate in Diabetic Rats*

It was of interest to compare the behavior of labeled acetate and pyruvate in phlorizinized animals with that of tryptophan.  $C^{14}H_3\cdot COONa$  and  $CH_3\cdot C^{14}OONa$  of high specific activity were diluted with inert acetate, as in the case of tryptophan. Phlorizinized and fasted rats were injected subcutaneously with 100 mg. (10  $\mu$ c. or  $1.25 \times 10^6$  counts/min.) of the compounds dissolved in 3 ml. of water. A third rat similarly received 111 mg. (10  $\mu$ c.) of  $CH_3\cdot C^{14}O\cdot COOH$ . The glucose and ketone bodies were analyzed as before, except that no attempt was made to collect the carboxyl  $CO_2$  of acetoacetate and  $\beta$ -hydroxybutyrate. The results are shown in Table III.

TABLE III

*Radioactivity of the Urinary Glucose and Ketone Bodies of Rats Injected with Labeled Acetate or Pyruvate*

Rat no.	Compound injected	Glucose			Acetone		
		Total mmoles	Counts/min./mmole	Per cent of dose	Total mmoles	Counts/min./mmole	Per cent of dose
5	$C^{14}H_3\cdot COONa$	3.92	20,900	6.55	2.72	6,520	1.42
6	$CH_3\cdot C^{14}OONa$	4.07	11,300	3.68	2.58	8,010	1.66
7	$CH_3\cdot C^{14}O\cdot COONa$	4.31	6,220	2.14	1.86	1,020	0.15

*Acetyl Formation from Tryptophan*

An adult male rat weighing 214 g. was given about 100 mg. of *p*-aminobenzoic acid by stomach tube. Approximately 11 mg. of tryptophan- $\beta$ -C<sup>14</sup> (9.4  $\mu$ c. or  $1.17 \times 10^6$  counts/min.) were injected intraperitoneally in 3 doses at intervals of 1 hr., the first injection being immediately after the administration of the *p*-aminobenzoic acid. Urine and expired CO<sub>2</sub> were collected for 24 hr. after the last injection and their activity determined as usual (Table IV).

TABLE IV  
*Distribution of Radioactivity in Rat After Administration  
of Tryptophan- $\beta$ -C<sup>14</sup>*

	Specific activity counts/min./mg.	Total counts/min.	Per cent of dose
Tryptophan (administered)	106,000	$1.17 \times 10^6$	100
Urine		$2.44 \times 10^5$	20.3
Expired CO <sub>2</sub> as BaCO <sub>3</sub>	24.6	$3.05 \times 10^5$	26.1
Acetyl- <i>p</i> -aminobenzoic acid	44.7		
Hemin	3.66		
Cholesterol digitonide	7.70		
Glycogen (muscle)	1.73		

The urine contained 18.5 mg. of acetyl-*p*-aminobenzoic acid as determined by the method of Marshall (13). To an aliquot of urine, containing 15 mg. of acetyl derivative, 150 mg. of the pure acetyl amino acid was added and reisolated by the method of Bloch and Rittenberg (14). The isolated compound was recrystallized to constant specific activity. It melted sharply at 250° and showed no depression in melting point when mixed with pure acetyl-*p*-aminobenzoic acid. Nitrogen content was 7.89% (theory, 7.82%). The radioactivity corrected for the carrier is recorded in Table IV.

At the end of 24 hr., the animal was killed by bleeding. Hemin was isolated from the red cells (15) and recrystallized (16). Cholesterol was obtained as the digitonide from about half of the liver (17). Glycogen was obtained from the muscle by the method of Stetten and Boxer (18). It was hydrolyzed by boiling in 1 *N* HCl and the acid removed *in vacuo*. Phenylglucosazone was prepared from the residue and recrystallized to constant specific activity (m.p. 204°; N = 15.83%). The radioactivities of these compounds are given in Table IV. As usual, the samples were counted long enough to reduce the error below 5%.

*Analysis of Protein Fraction*

The liver, intestines, stomach, kidney, and spleen of the rat used in the acetylation study were pooled, homogenized, and treated with 10% trichloroacetic acid. The precipitated protein was washed 3 times with 5% trichloroacetic acid solution containing about 50 mg. tryptophan, and then once with the acid alone. The lipides were

extracted with hot alcohol, alcohol-ether mixture and finally with ether. The dried protein (2.25 g.) had 69.1 counts/min./mg. (total activity:  $1.5 \times 10^6$  counts/min. or 12.5% of dose).

### *Tryptophan*

Five hundred mg. of the protein was hydrolyzed by refluxing for 5 hr. with 10 ml. of 5 N NaOH. Tryptophan was precipitated from the hydrolyzate as a complex with mercuric sulfate according to Block and Bolling's adaptation (19) of the Millon-Folin method. The precipitate was dissolved in 1 N HCl and the tryptophan estimated on an aliquot by the color developed with Folin's phenol reagent (19). The total tryptophan content was 6.90 mg. or 1.38% of the protein. Mercury was removed from the solution with hydrogen sulfide, and sulfate with barium hydroxide. The bulky precipitate of barium sulfate was washed repeatedly with boiling water containing in all 60 mg. of DL-tryptophan. The washings were mixed with the original solution containing the tryptophan and evaporated to dryness in vacuum. The residue was dissolved in a small amount of glacial acetic acid and benzene was added to it. Tryptophan acetate (salt) separated out almost quantitatively and was analyzed for radioactivity and nitrogen ( $N = 10.2\%$ ; theory = 10.6%).

The tryptophan acetate was allowed to react with formaldehyde in the cold (20) and the 3,4,5,6-tetrahydro-4-carboline-5-carboxylic acid isolated. This compound is easily obtained and readily recrystallized as shiny leaflets. It melted with effervescence sharply at  $310^\circ$  (reported  $310^\circ$ ), gave the same mixed melting point and showed no change in specific activity on recrystallization ( $N = 12.8\%$ ; theory = 12.96%). The analytical data on the isolated tryptophan are presented in Table V.

TABLE V

*Distribution of  $C^{14}$  Activity of the Proteins in the Constituent Amino Acids*

Compound	Total counts/min.	Relative activity
Protein	34,550	100
Tryptophan	21,000	60.8
Dicarboxylic acids	11,150	32.3
Serine (beta-carbon)	187	0.54
Unknown	—	6.36

### *Dicarboxylic Acids*

Five hundred mg. of the protein was hydrolyzed by boiling under reflux with 20% HCl for 20 hr. Acid hydrolysis destroys tryptophan. The hydrolyzate was evaporated to dryness *in vacuo* and the residue decolorized with charcoal. It contained 38.1% of the radioactivity of the protein. The basic amino acids were removed from the hydrolyzate by treatment with phosphotungstic acid (21). The excess reagent was extracted with a mixture of amyl alcohol and ether, and the amino acid solution evaporated to dryness *in vacuo*. The calcium salts of the dicarboxylic acids were prepared according to the method of Bailey *et al.* (22) and purified by reprecipitation. Calcium was removed with oxalic acid and an aliquot of the residue evaporated on a plate and analyzed. The aspartic and glutamic acids were not separated.

*Serine*

The protein hydrolyzate free from basic and acidic amino acids was next oxidized with periodic acid (23) and the formaldehyde formed from the beta-carbon of serine distilled over and obtained as methylene-bis-methone. It was found to be weakly radioactive (Table V). To confirm the result, the oxidation was repeated with a fresh hydrolyzate according to the procedure of Rees (24) and the formaldehyde recovered from the distillate. The methone was pure by the standards mentioned before. Calculating back from its specific activity, the serine (beta-carbon) had 5 counts/min./mg.

The remainder of the protein radioactivity (6.36%) was not accounted for, but it is conjectured that alanine is responsible for most of the remaining activity.

## DISCUSSION AND CONCLUSIONS

The glucose excreted by the rats given radioactive tryptophan had high specific radioactivity (Table I). Degradation of the urinary glucose showed that 50% of the  $C^{14}$  isotope was in Carbons 1-3 (Table II). This is consistent with the idea that glucose is a symmetrically-labeled molecule formed by the condensation of two units of pyruvate by the reversal of the reactions of glycolysis.

Carbon 6 contained 25% of the total activity of the urinary glucose (Table II). We have no direct evidence to show whether the remaining 25% of the activity was in Carbon 4 or 5 or distributed between them. In order to obtain complete information of the distribution, this method has to be used in conjunction with any of the other known methods of glucose degradation.

The distribution of the isotope in glucose arising from the administration of different compounds is outlined below:

Source	Labeled carbon	Reference
$C^*O_2$	3, 4	(25)
$CH_3 \cdot C^*OOH$	3, 4	(26)
$C^*H_3 \cdot COOH$	1, 2, 5, 6	(26)
$CH_3 \cdot CO \cdot C^*OOH$	3, 4	(27)
$CH_3 \cdot C^*O \cdot COOH$	2, 5	(27)
$C^*H_3 \cdot CO \cdot COOH$	1, 6	(27)

The distribution of  $C^{14}$  that we have found in the excreted glucose can be explained by the formation of  $C^{14}H_3 \cdot COOH$  or similar derivative from the administered tryptophan. More direct evidence on the inclusion of the beta-carbon of tryptophan into an acetyl group is available from the acetylation experiment (Table IV). The data on the

distribution of  $C^{14}$  in the glucose resulting from  $C^{14}H_5 \cdot COOH$  (Table II) are in agreement with previous work (26) as far as the method permits.

It is noted from Table III that the per cent conversion of  $C^{14}$  of the carboxyl-labeled acetate to glucose is about half that of the methyl-labeled acetate. This result is to be anticipated if the animals were metabolically equivalent. One-half of the  $C^{14}$  of the carboxyl-labeled acetate is lost in the formation of pyruvate from oxalacetate after passing through the tricarboxylic acid cycle. The lower conversion of pyruvate  $C^{14}$  to acetoacetate compared to acetate conversion may be expected in phlorizinized animals. Although other differences are evident from Table III, they cannot be satisfactorily explained from present knowledge.

The labeled beta-carbon of tryptophan appeared in acetyl-*p*-amino-benzoic acid (Table IV) confirming acetyl formation from tryptophan. Bloch and Rittenberg (14) determined from the dilution of fed  $C^{13}$ -acetate that at least 15–20 mmoles of acetic acid or acetyl derivative are formed daily per 100 g. of rat tissue. Assuming this value, the fraction of the administered tryptophan that was converted to acetyl derivative in the animal may be calculated. Since acetyl-*p*-amino-benzoic acid had 44.7 counts/min./mg., the acetic acid in it should have contained 133 counts/min./mg. The total activity in the acetyl pool of the animal was, therefore,  $2.56 \times 10^5$  to  $3.4 \times 10^5$  counts/min. This shows that about 21–28% of the administered  $C^{14}$  could have appeared in the form of a two-carbon unit. The value is in good agreement with the amount oxidized and recovered as  $CO_2$  (26.1%) and indicates that the major pathway of oxidation of the tryptophan side chain is via an intermediary acetyl derivative.

Acetate is biologically very reactive and forms among other things, hemin (14) and cholesterol (28). The introduction of the labeled beta-carbon of tryptophan into hemin and cholesterol (Table IV) is further evidence of acetyl formation in tryptophan catabolism. Before it was discovered that the porphyrin molecule is synthesized in the organism from compounds like acetate and glycine (29), it was believed that the indole ring of tryptophan might be the precursor. Recent work with tryptophan labeled with  $N^{15}$  in the indole, however, shows that tryptophan is not a direct precursor of hemin (30).

The appreciable radioactivity in the dicarboxylic amino acids of the proteins (Table V) probably resulted from the subsequent oxidation

pathway of the labeled acetyl derivative. Rittenberg and Bloch (31) and Greenberg and Winnick (32) observed that feeding isotopically-labeled acetate led to the labeling of the glutamic and aspartic acids in the animal.

The tryptophanase reaction resulting in the formation of indole and pyruvic acid (33) does not appear to be significant in the rat in view of the observed  $C^{14}$  distribution in glucose and low activity of alanine. There was slight but definite radioactivity in serine. This observation is interesting though of little quantitative importance. No definite inference can be drawn about the mode of formation of the serine although a possible mechanism is the reversal of the serine to pyruvate reaction (34).

A tentative scheme to represent the catabolism of tryptophan is outlined in Fig. 1 showing the formation of *o*-amino-*m*-hydroxy-benzoylactic acid as an intermediate. This is a beta-ketoacid and can break down to a two-carbon derivative (as probably happens with

#### Pathway of Tryptophan Catabolism

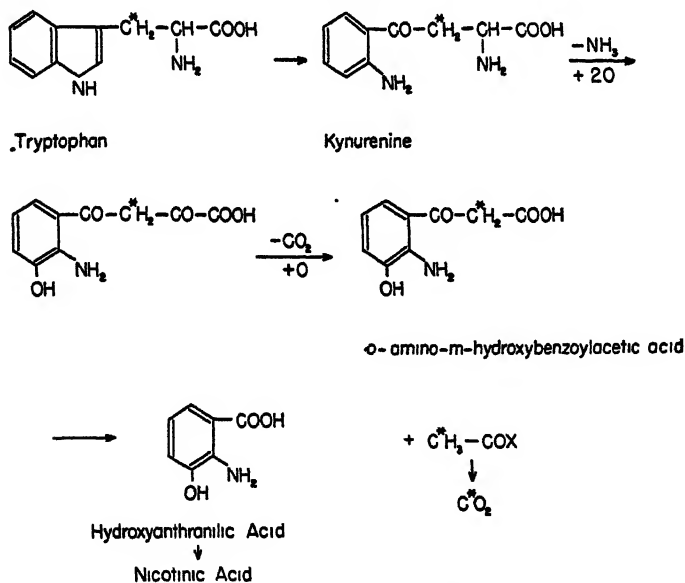


FIG. 1. Pathway of tryptophan metabolism.

acetoacetate) and hydroxyanthranilic acid. In agreement with Fig. 1, Heidelberger and co-workers (1, 2) showed that the beta-carbon of tryptophan did not appear in nicotinic acid and that Carbon 3 of the ring was changed to the carboxyl of nicotinic acid. Hydroxyanthranilic acid has been shown to be a precursor of nicotinic acid (35) and has been isolated from filtrates of *Neurospora* mutant cultures (36).

The transfer of the C<sup>14</sup> of the  $\beta$ -carbon into acetyl-*p*-aminobenzoic acid, hemin, and cholesterol (Table IV), and its distribution in glucose is evidence that a two-carbon (acetyl) unit is a product of the catabolism of the side chain of tryptophan. No C<sup>14</sup> was detected, however, in the isolated ketone bodies. In view of the extensive evidence in favor of the conversion of acetate to acetoacetate and vice versa in the intact animal (37-39) this observation is difficult to explain. A fuller understanding must await more detailed information.

It may be pointed out that the catabolic scheme given in Fig. 1 explains satisfactorily the observations of Borchers, Berg, and Whitman (6). They noted that tryptophan decreased ketonuria markedly. Substances which reduce ketone body excretion are also generally glycolytic. But tryptophan was unusual in that it did not increase liver glycogen of fasted rats. The acetyl derivative arising from tryptophan via the beta ketoacid may not recombine to form ketone bodies but in its oxidation will spare an equivalent amount of fatty acid oxidation thereby decreasing ketonuria. Thus, an effect analogous to feeding carbohydrates is produced by tryptophan administration.

It appears, therefore, necessary to recognize that certain substances which in their catabolism produce a beta-ketoacid different from acetoacetic acid may form acetyl groups without increasing ketonuria in diabetic animals. On the other hand, the result may be a decrease in ketonuria similar to that obtained in carbohydrate feeding.

#### SUMMARY

Tryptophan labeled with C<sup>14</sup> in the beta position was administered to phlorizinized rats. The glucose excreted in the urine was radioactive. The distribution of the radioactivity in the glucose, determined by oxidation of phenylglucosotriazole with periodate, suggested the intermediary formation of a methyl-labeled acetyl derivative from tryptophan. However, there was no radioactivity in the ketone bodies. Intermediary formation of acetate was confirmed by the appearance of the C<sup>14</sup> in acetyl-*p*-aminobenzoic acid, hemin, and cholesterol.

Analysis of the proteins of an animal administered the labeled tryptophan showed that tryptophan and dicarboxylic acids accounted for most of the activity. Serine had slight activity. It is suggested that in the breakdown of tryptophan a beta-ketoacid, *o*-amino-*m*-hydroxybenzoylactic acid, may be formed which decomposes to yield hydroxyanthranilic acid and an acetyl derivative. A decrease in ketonuria in a diabetic animal resulting, in certain cases, from a formation of acetyl groups is discussed.

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# Liberation of Amino Acids and Peptides from Raw and Heated Bovine Plasma Albumin by Pepsin and Trypsin <sup>1</sup>

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## INTRODUCTION

In earlier work it was shown that the liberation by pancreatin of amino acids from soybean meal in a form utilizable by microorganisms was increased by optimum heating and decreased by prolonged heating, and that these effects could be correlated with growth rates obtained with chicks (1, 2, 3). However, the liberation of amino acids from casein by crude preparations of pepsin, pancreas, and erepsin was relatively unaffected by heat (4). Among the difficulties encountered in the previous studies were incomplete solution of the protein due to the use of denatured, impure proteins and the use of crude enzyme preparations. The present study was undertaken to determine the extent of liberation of amino acids and peptides by crystalline pepsin and trypsin from crystalline bovine plasma albumin in the raw state and after heating, when conditions which favored solubility of the albumin were used.

## EXPERIMENTAL

### *Effect of Heat on Solubility*

Bovine plasma albumin (Armour's) is readily soluble in 2% concentration in water. Such a solution has a cloudy appearance and a pH of 4.5. If acid or alkali is added to pH 1 or pH 10, respectively, a clear solution is formed, although a cloudy suspension results if the pH is readjusted to within 3.5 to 4.8—the isoelectric range. Hence, this protein is ideal for digestion experiments with pepsin and trypsin at their

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respective pH optima. Furthermore, it is well characterized with respect to amino acid content and homogeneity and is available in quantity for experimental purposes. The sample used for these experiments contained 3.5% moisture, 0.2% ash, and 15.9% nitrogen determined by the Kjeldahl method.

Since the protein is an albumin, difficulty was expected in attempting to solubilize samples which had been heated. When autoclaved as a solid or in a 2% water solution for only 4 min. at 15 lb. pressure, it did not dissolve after shaking for 24 hr. even in the presence of very dilute alkali at pH 8. More concentrated alkali, *i.e.*, 1 *N*, gradually dissolved the heated protein, but high salt concentrations were unsatisfactory for subsequent microbiological assays.

Results of attempts to maintain solubility by autoclaving with added acid or alkali are summarized in Table I. Two and a half per cent solutions of bovine plasma albumin were autoclaved at 15 lb. for varying lengths of time and at varying pH. The protein was then solubilized by adding 1 *N* sodium hydroxide, and diluted to 2%

TABLE I

*Solubility of 2.5% Bovine Plasma Albumin Solutions Autoclaved at 15 lb. for Varying Lengths of Time and at Varying pH*

Time of autoclaving min.	pH of solution <sup>a</sup>			
	2	4.5	8	11
0	Clear solution	Cloudy solution	Clear solution	Clear solution
4	Soluble; slightly cloudy	Insoluble	Soluble	Soluble
30	Soluble	Insoluble	Soluble	Soluble; amber color
120	Soluble	Insoluble	Soluble; slightly amber color	Soluble; amber color
900	Soluble	Insoluble	Viscous; amber color	Soluble; amber color

*Milliliters of Nitrogen Gas Produced in Nitrous acid Amino Nitrogen Determination<sup>b</sup>*

A 2-ml. aliquot of solution which had been solubilized with 1 *N* NaOH prior to dilution of 2% was used.

hr.				
0	—	0.84	—	—
2	1.65	0.81	0.80	1.10

<sup>a</sup> pH was adjusted by addition of acid or alkali prior to autoclaving. Pure albumin dissolved in water is pH 4.5.

<sup>b</sup> Temperature 26°C. Pressure 740 mm. Hg.

prior to determination of amino nitrogen by the micro-nitrous acid method of Van Slyke (5). It is apparent that acid or alkaline conditions are necessary to maintain solubility of bovine plasma albumin during heating. Since slight hydrolysis occurred at pH 2 and hydrolysis plus decoloration occurred at pH 11, while neither was found at pH 8 when autoclaved up to 30 min., the latter conditions were chosen for succeeding work.

### *Enzyme Hydrolysis*

The concentrations of crystalline pepsin and trypsin (Armour's) required for suitable rate and extent of hydrolysis of raw bovine plasma albumin were next determined. Two and one-half per cent solutions of albumin in water were prepared and adjusted to pH 2 with concentrated hydrochloric acid for digestion by pepsin, and to pH 8 with concentrated sodium hydroxide for digestion by trypsin. The solutions were diluted to 2%, saturated with toluene, and allowed to reach 37°C. in an air incubator. Solutions of pepsin in water and trypsin (double quantity of sample containing 50% magnesium sulfate) in 0.01 *N* sodium hydroxide were added to albumin solutions at 15-min. intervals to supply final concentrations of 0.01–2% of enzyme to substrate (see Fig. 1). The enzyme solutions were made up at 100 times the final concentrations to avoid significant dilution when added to the albumin solutions. Two-ml. aliquots were removed immediately after addition of the enzyme, and after 1, 2, 6, 12, 24, and 48 hr. for immediate amino nitrogen determinations by the micro-nitrous acid method of Van Slyke (5). After 24 hr., half the 0.1% peptic digest was immediately adjusted to pH 8 with concentrated sodium hydroxide, and half the 1% tryptic digest was immediately adjusted to pH 2 with concentrated hydrochloric acid. Concentrated solutions of trypsin and pepsin, respectively, were added to these digests, and aliquots were analyzed as previously for amino nitrogen during the hydrolysis. The solutions were kept saturated with toluene to prevent spoilage. The results of this experiment are plotted in Fig. 1. Enzyme to substrate concentrations of 0.1% pepsin/albumin and 1% trypsin/albumin were chosen for subsequent work since they produced rates and extents of hydrolysis which were measurable but still not too high to be independent of enzyme concentration.

The effect of the length of time of autoclaving on the digestibility of bovine plasma albumin by pepsin and trypsin was determined in the next experiment. The conditions of autoclaving and the subsequent digestions were done as previously described except that alpha amino nitrogen was measured immediately after removal of 5 ml. of digest by formol titration of carboxyl groups rendered titratable by the formol reaction. The titration was done with 0.053 *N* sodium hydroxide under nitrogen bubbled into a conical flask after adding 1 ml. of neutralized 40% formol and 2 drops of octyl alcohol as recommended by Mellander (6). The digests were titrated to pH 9.5 with a pH meter using a glass electrode in the titration flask (7). Since autoclaving at pH 8 at 15 lb. for 30 min. produced a decided alteration in digestibility by trypsin without noticeable browning, coagulation, or hydrolysis of the substrate (see Table I), these conditions of heating were used for the final digestion experiment.

In this experiment aliquots of 24-hr. peptic and tryptic digests, and 48-hr. peptic-tryptic and tryptic-peptic digests of raw and autoclaved albumin, were heated in a boiling water-bath for 10 min. to inactivate the enzymes, and stored at -4°C. Total free amino acids were determined by the manometric micro-ninhydrin procedure

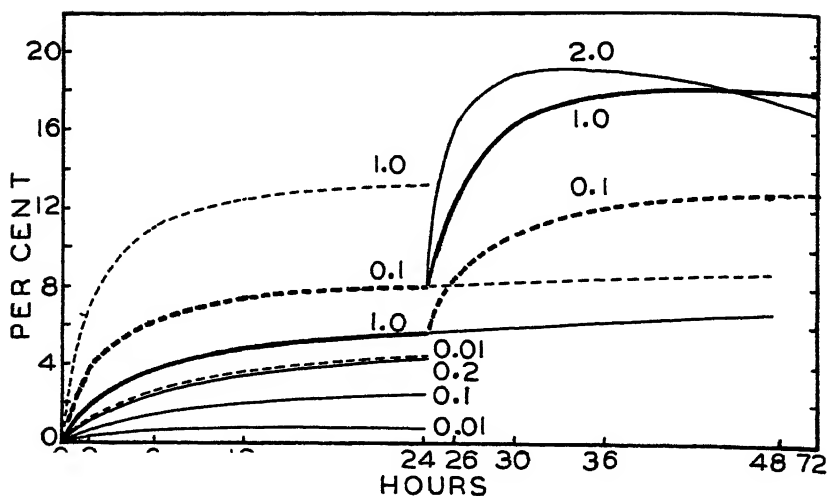


FIG. 1. Per cent release of amino nitrogen from raw bovine plasma albumin digested with varying levels of pepsin (broken line) at pH 2 and trypsin (solid line) at pH 8. Numbers within the figure refer to per cent enzyme with respect to substrate. Heavy lines represent conditions chosen for the final experiment for amino acid analysis.

(2 ml. carbon dioxide gas) of Van Slyke *et al.* (8) using 5 ml. of digest, 100 mg. ninhydrin, 100 mg. pH 2.5 buffer, and a boiling time of 20 min.

Total amino nitrogen was determined according to the manometric microprocedure (2 ml. nitrogen gas) of Van Slyke (9) using 5 ml. of digest.

### Amino Acid Assays

The digests were assayed microbiologically for 18 amino acids. Since lower values were obtained with *Leuconostoc mesenteroides* P-60 in previous work with pancreatic digests than a number of other organisms, it was used for as many amino acids as possible in an attempt to avoid excessive peptide activity. This organism was used to determine aspartic acid, glycine, isoleucine, leucine, lysine, phenylalanine, serine, tryptophan, and tyrosine; a related organism, *Leuconostoc citrovorum* 8081, was used for alanine, arginine, cystine, glutamic acid, histidine, methionine, proline, threonine, and valine. The media were similar to those of Henderson *et al.* (10) except for the following modifications: The level of asparagine in the glutamic acid medium was reduced to one-fourth that formerly used to prevent initial lag in acid production. Lilly's reticulogen was added to all media for *Leuc. citro-*

*vorum* as recommended by Steele *et al.* (11). Pyridoxine was used as the sole form of vitamin B<sub>6</sub> in media for alanine and serine at 20 times the level of pyrodoxal used for other amino acids (11, 12). The tubes were autoclaved no longer than 10 min. at 15 lb. (121°C.).

Release of amino acids and peptides from the enzyme digests was compared with the total release by acid or alkaline hydrolysis. Tryptophan and tyrosine were assayed using hydrolyzates prepared by autoclaving 1 g. of albumin with 20 ml. of 5 *N* sodium hydroxide at 15 lb. for 15 hr. (10). The values obtained were doubled since racemization occurs under these conditions. Cystine was assayed using a hydrolyzate prepared by autoclaving 1 g. of albumin with 40 ml. of 2 *N* hydrochloric acid at 15 lb. for 3 hr. (4). Assay of other amino acids, determination of total amino acids by the manometric ninhydrin method, and determination of total amino nitrogen by the manometric nitrous acid method were done using albumin hydrolyzed with 40 ml. of 3 *N* hydrochloric acid per gram of protein at 15 lb. for 5 hr. (10). A quantity of hydrolyzate containing 100 mg. of protein was also analyzed for ammonia nitrogen by making it alkaline with excess 40% sodium hydroxide and distilling into standard acid as in the final stages of a semimicro-Kjeldahl total nitrogen determination.

## RESULTS AND DISCUSSION

It is seen in Table I that bovine plasma albumin is rendered soluble during heating in the presence of excess acid or alkali. When large amounts of acid or alkali are used, *i.e.*, pH 2 and pH 11, this solubilizing effect is accompanied by measurable amounts of hydrolysis. However with only a trace of alkali, *i.e.*, pH 8, the protein remains in solution without any detectable hydrolysis when autoclaved for periods up to 2 hr. A soluble "sodium albuminate," *cf.* sodium caseinate, is formed which upon prolonged heating becomes more viscous and amber in color. For this study the protein was autoclaved for only 30 min. to avoid apparent changes such as coagulation, browning, or increase in viscosity. At pH 2, hydrolysis appears to be the main solubilizing effect. At pH 10, hydrolysis prevents the increase in viscosity noted at pH 8 and destructive changes appear since an amber color is produced rather early.

The digestibility of raw bovine plasma albumin by various amounts of pepsin and trypsin is shown in Fig. 1. Pepsin was found to be 20

times as active as trypsin on a weight basis for the hydrolysis of native albumin. A 0.1% ratio of pepsin to albumin and a 2% ratio of trypsin to albumin each produced superimposable hydrolysis curves, or about 8% hydrolysis after 24 hr. It should be noted that when trypsin was used on a peptic digest, more hydrolysis due to trypsin occurred than when it acted on the native protein. Apparently pepsin renders some linkages more accessible to attack by trypsin than in the native protein. This is contrary to the supposition that digestion by pepsin and trypsin is merely additive when used successively. This effect was not noted when a tryptic digest was hydrolyzed by pepsin.

In Table II are shown the effects of heat upon the digestibility of bovine plasma albumin by pepsin and trypsin. Autoclaving the protein at pH 8 at 15 lb. for 5 min., 30 min., or 2 hr. had only a slight effect upon the digestibility by pepsin. Autoclaving for 30 min. increased the digestibility by trypsin, while autoclaving for only 5 min. or for 2 hr. had little effect upon the digestibility by this enzyme. It should be pointed out that in this experiment the extent of hydrolysis by trypsin tended to decrease towards the end of the digestion period when raw protein was used. This effect was noted frequently in repeat experiments (see Fig. 1, 2% trypsin) but rarely when heated albumin was used. Changes in pH generally correlated with the extent of hydrolysis by each enzyme.

When pepsin was followed by trypsin, the rate and extent of digestion contributed by the latter enzyme was uniform and independent of previous heat treatment of the substrate. Thus preliminary digestion of raw albumin by pepsin renders it nearly as digestible by trypsin as albumin which had been autoclaved for 30 min. However the action of pepsin on tryptic digests was slightly decreased by heat. The total digestion by the trypsin-pepsin sequence was less than the reverse combination in all cases except when the protein had been autoclaved for 30 minutes—the optimum heat treatment for maximum digestion of native protein by trypsin. It is apparent that the physiological combination, pepsin followed by trypsin, produces hydrolysis which is the most independent of previous heat denaturation of the protein.

The amino acid values obtained using acid and alkaline hydrolyzates (Table III) agree favorably with those previously reported by Henderson *et al.* (10). In this study the content of four additional amino acids—alanine, cystine, glycine, and serine—is reported. While assays for these amino acids are not quite as reliable as the others, the values are

included for comparison with other amino acids of the release by pepsin and trypsin. If the per cent contributed by water of hydrolysis of each amino acid is subtracted from the total, 113.1, a value of 95.3% is obtained for the total amino acid residues. Similarly, the nitrogen of all amino acids determined accounts for 90.5% of the total nitrogen determined by the Kjeldahl method. Since 5.9% of the total nitrogen

TABLE II

*Digestion of Bovine Plasma Albumin Autoclaved at 15 lbs. for Varying Lengths of Time by Pepsin Followed by Trypsin and Trypsin Followed by Pepsin<sup>a</sup>*

Digestion time	Enzyme	Autoclaving time				Enzyme	Autoclaving time			
		None	5 min.	30 min.	2 hr.		None	5 min.	30 min.	2 hr.
1	Pepsin	0.72	0.70	0.58	0.69	Trypsin	0.40	0.60	0.97	0.42
2		0.87	0.84	0.67	0.80		0.52	0.75	1.30	0.63
6		1.36	1.25	1.01	0.91		1.15	1.15	1.63	0.85
24		1.44 (2.65)	1.40 (2.7)	1.47 (2.7)	1.41 (2.65)		0.52 (6.9)	1.05 (6.6)	1.60 (6.6)	0.80 (6.8)
2	Trypsin (preceded by pep- sin)	1.11	1.03	0.96	0.89	Pepsin (preceded by tryp- sin)	0.66	0.55	0.57	0.45
6		1.12	1.24	1.03	1.07		1.04	1.10	0.72	0.87
24		1.39 (7.15)	1.37 (7.2)	1.34 (7.2)	1.30 (7.2)		1.32 (2.6)	1.09 (2.4)	0.95 (2.3)	1.07 (2.2)
26		1.98	1.87	1.63	1.69		1.18	1.30	1.87	1.08
30	Totals	2.48	2.49	2.04	1.98	Totals	2.19	2.25	2.35	1.72
48		2.83	2.77	2.71	2.71		1.84	2.14	2.55	1.87

<sup>a</sup> The figures in parentheses represent the pH of each solution after digestion by the enzyme. Starting pH was 2 for pepsin and 8 for trypsin. Remaining figures are milliliters of formol titer of 5 ml. of hydrolyzate using 0.053 N NaOH.

All values have been corrected by subtracting titrations done immediately after addition of the enzyme.

released by acid hydrolysis was ammonia nitrogen, 96.4% of the total nitrogen is accounted for. The remainder is probably due to undetermined amino acids and/or to losses of certain amino acids during acid and alkaline hydrolysis. Since the total accountable nitrogen is higher than the total amino acid residues, it is also possible that some of the ammonia arose not from the amide nitrogen of the dicarboxylic amino acids, but also as a result of deamination of other amino acids. Gortner

and Holm (13) have stated that this can occur after prolonged hydrolysis. This could cause certain amino acids to be partially inactive toward microbiological assay organisms, resulting in a low amino acid total as well as a high ammonia-nitrogen value. Amino nitrogen determined by the volumetric nitrous acid method, the manometric nitrous acid method, and the manometric ninhydrin procedure account for 75, 73, and 65% of the total nitrogen, respectively. Apparently, after acid hydrolysis, some unaccounted for amino nitrogen is determined by the nitrous acid method which is not measured by the ninhydrin reagent.

The percentage release of amino acids from bovine plasma albumin by pepsin and trypsin is shown in Table III. Figures for each individual amino acid represent any amino acid and/or peptides utilized in place of the listed amino acid by the microorganism used to assay the amino acid. All figures are based upon totals obtained with acid or alkaline hydrolyzates. It is assumed that no decreases in amino acid content resulted when the protein was autoclaved for 30 min. at pH 8. The total *free* amino acids released by pepsin and trypsin determined by the manometric ninhydrin method were negligible. In the case of trypsin this is in agreement with the results of Van Slyke which showed that no release of free amino acids occurred if the trypsin preparation was pure (8). Comparison with the total release of amino groups as measured by the nitrous acid procedure indicates that the bulk of the hydrolysis consisted of release of peptides with an average size of 11.5 amino acid residues for pepsin, and from 17.5 to 12.5 amino acid residues for trypsin. In view of the negligible release of free amino acids, and, since these enzymes are proteinases, it is surprising that the release of peptides containing amino acids utilized by the assay organisms is approximately twice that of amino nitrogen. This indicates utilization of rather large peptides of many amino acids by the microorganisms used for assay. In fact it would appear that an unusual amount of stimulation by these peptides occurred accounting for the excess activity. It is also possible that duplication in peptide activity occurs since a given peptide may be active for a number of individual amino acids it contains, while each peptide is measured only once by the nitrous acid amino nitrogen method. Growth stimulation of peptides in excess of that predicted from amino acid content (activity of more than 100%) could account for the "drift" noted in a number of assays: namely, arginine, aspartic acid, glycine, isoleucine, serine, and tryptophan. However, it would fail to explain a perfectly linear growth

TABLE III

*Per Cent Release<sup>a</sup> of Peptides Utilized Microbiologically as Amino Acids, and Per Cent Release of Free Amino Acids and Amino Nitrogen from Raw and Heated Bovine Plasma Albumin by Pepsin and Trypsin*

Amino acid	Content <sup>b</sup>	Pepsin 24 hr.		Trypsin 24 hr.		Pepsin, 24 hr., followed by trypsin, 24 hr.		Trypsin, 24 hr., followed by pepsin, 24 hr.	
		Raw	Heated	Raw	Heated	Raw	Heated	Raw	Heated
Alanine	<i>per cent</i> (8.80)	12.5	13.0	4.2	8.0	27	30	17	25
Arginine	5.84	13.1	15.3	15.4	35.0	73	66	35	69
Aspartic acid	11.1	1.6	1.9	0.2	0.0	6.0	7.0	3.1	6.3
Cystine	(5.70)	0.0	0.5	0.1	0.0	12	23	4.1	8.4
Glutamic acid	17.6	20.0	19.5	4.7	11.2	37	39	23	36
Glycine	(1.82)	6.1	5.9	0.0	0.0	13	11	3.3	5.2
Histidine	4.07	7.9	7.4	7.2	16.1	44	42	5.7	36
Isoleucine	3.24	10.3	10.3	0.0	0.0	30	27	11	16
Leucine	11.3	16.5	15.4	3.8	8.5	29	24	18	22
Lysine	10.9	0.0	0.0	5.9	14.3	2.8	2.8	7	17
Methionine	0.91	32.0	33.0	12.5	19.3	48	52	41	55
Phenylalanine	6.51	14.5	14.3	0.8	1.9	27	24	17	19
Proline	5.23	13.0	13.5	3.5	9.0	29	27	19	32
Serine	(5.70)	11.8	13.6	2.2	5.1	15	16	11	12
Threonine	5.80	27.0	28.0	5.7	23.0	64	54	34	47
Tryptophan	0.55	8.7	8.5	14.7	17.3	38	34	19	27
Tyrosine	4.27	40	40	9.6	18.8	70	66	57	68
Valine	4.25	68	51	9.0	22.0	95	94	66	94
Average <sup>c</sup>	113.1 <sup>c</sup>	16.0	15.5	5.1	14.7	29.2	32.9	20.8	41.9
Free amino acids (ninhydrin)	65 <sup>d</sup>	0.60	0.55	1.00	0.90	1.80	1.80	1.55	1.75
Aminonitrogen (nitrous acid)	73 <sup>e</sup>	8.7	8.7	5.8	8.0	17.5	18.2	12.8	18.5

<sup>a</sup> Actual amounts released by enzymes divided by content, the fraction then multiplied by 100.

<sup>b</sup> Per cent in moisture-free, ash-free bovine plasma albumin after hydrolysis.

<sup>c</sup> Arithmetic total.

<sup>d</sup> Per cent carboxyl N of total N.

<sup>e</sup> Per cent amino N of total N.

response in the case of the other amino acids, or the lack of reproducibility of "drift" in repeat assays.

It is of interest that greater utilization of peptides was noted in this study than with casein using crude pepsin and pancreatin (4). The

ratio of amino acids to amino nitrogen was 0.5 with pepsin and 1 with pancreatin using casein, compared with 2 for crystalline pepsin or trypsin using bovine plasma albumin. This may be due in part to the use of different microorganisms for the assay of amino acids in this study. Ågren (14) recently found greater utilization of leucine peptides by *Leuc. citrovorum* than by *Leuc. mesenteroides*. The suggestion has been made by Simmonds *et al.* (15) that the utilization of peptides by microorganisms may be preceded by enzymatic hydrolysis to yield essential amino acids. If this is true, it would seem probable that the enzymes involved must be extracellular, unless the peptides which are utilized pass through the bacterial membrane.

The average release by pepsin of utilizable peptides as well as the release of peptides active for each amino acid were unaffected by previous heating of the albumin. However, heating increased the release of active peptides by trypsin. The average release of utilizable peptides after treatment by pepsin followed by trypsin was higher than that released when each enzyme acted on the protein initially and was unaffected by previous heating. However the release by trypsin followed by pepsin was lower from the raw protein, although the release obtained from the heated protein with this enzyme combination approached that obtained with the reverse enzyme sequence with both the raw and heated proteins. It follows that maximum extent of digestion occurred using the pepsin-trypsin sequence or the trypsin-pepsin sequence provided that in the latter case the protein had been previously denatured by heat. Heat denaturation and pepsin each render bovine plasma albumin more readily digestible by trypsin; however, the action of pepsin nullifies any previous beneficial effects gained by heat denaturation. These results agree with those summarized in Table II.

The results obtained with trypsin support the hypothesis advanced by Linderstrøm-Lang, Hotchkiss, and Johansen (16) that denatured proteins contain altered peptide linkages which are more readily attacked by proteinases. However, it would appear that this may not be true for all proteinases since action by pepsin was shown to be relatively unaffected by heating the substrate. Furthermore, it seems probable that there may be more than one denatured form since variable tryptic digestion is obtained if the substrate is heated for varying lengths of time.

Considerable variation was found in the extent of the release of peptides containing amino acids utilized by the microorganisms.

Peptides containing utilizable aspartic acid, cystine, and lysine were not liberated to any significant extent by pepsin. Those containing available tyrosine, valine, and methionine were released to the greatest extent by this enzyme. Utilizable aspartic acid, cystine, glycine, isoleucine, and phenylalanine were not released by trypsin, while arginine, methionine, and tryptophan were rendered most available to microorganisms. Pepsin liberated larger quantities than did trypsin of peptides active for all amino acids except the basic amino acids and tryptophan; these were released in more available form by the latter enzyme. This is in general agreement with the known specificity of trypsin with respect to synthetic substrates. Typical substrates for this enzyme usually contain lysine or arginine residues. Up to 70% of the tyrosine and 95% of the valine present in peptides released by pepsin followed by trypsin were utilized by *Leuc. mesenteroides* and *Leuc. citrovorum*, respectively. These results are in general agreement with those previously reported with casein.

#### SUMMARY

A study was made of the liberation of amino acids and peptides by crystalline pepsin and trypsin from crystalline bovine plasma albumin in the raw state and after heating using conditions which favored solubility of the protein throughout the period of heat treatment. It was found that:

1. Heat treatment has no effect on the digestibility of bovine plasma albumin by pepsin. Either heating or predigestion by pepsin renders the albumin more digestible by trypsin.
2. Pepsin followed by trypsin is better than trypsin followed by pepsin in obtaining maximum digestion of raw albumin.
3. Pepsin and trypsin release negligible quantities of free amino acids from raw or heated albumin.
4. In the quantities used for optimum and measurable rates of hydrolysis, pepsin liberates larger quantities than does trypsin of peptides microbiologically active for all amino acids except the basic amino acids and tryptophan; these are released in more available form by the latter enzyme.
5. *Leuconostoc mesenteroides* P-60 and *Leuc. citrovorum* 8081 utilize peptides released by pepsin and trypsin.

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# The Colorimetric Determination of Adenine <sup>1</sup>

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## INTRODUCTION

Hutchings *et al.* (1) described a method for the estimation of folic acid by reducing with zinc in acid solution, diazotizing the resulting amine and coupling with the reagent of Bratton and Marshall (2), *N*-(1-naphthyl) ethylenediamine hydrochloride. When this reaction was applied to tissue extracts by Glazko and Wolf (3) they observed that the presence of adenine introduced an interfering color while various other purines and pyrimidines did not give colored solutions.

Advantage has been taken of these observations to demonstrate that the colored complex with adenine can be used for the micro determination of this substance in pure solution or in hydrolyzates of nucleotides or nucleic acids.

## METHOD

### *Conditions for Reduction and Diazotization*

The purine was reduced in 1 *N* HCl or H<sub>2</sub>SO<sub>4</sub> solution by the addition of zinc dust for a period of 30 min. at 90°C. Zinc foil or granulated zinc was much less effective. Hutchings *et al.* (1) observed that for folic acid the optimum reduction time at room temperature was 10 min., some destruction of the "aromatic amine" occurring after longer periods. They also found that a trace of gelatin helped to stabilize the amine and that the color of the coupled compound was sensitive to light.

Adenine required a much longer period of reduction and only after 2 hr. at 18°C. with constant shaking was a reproducible intensity of magenta color obtained with concentrations of 5–40  $\mu$ g. adenine/ml. of solution. The color was stable for several hours and not very sensitive to light. It was found, however, that slightly greater color production was obtained by reducing for 30 min. at 90°C. and therefore the following technique was adopted for quantitative analysis.

<sup>1</sup> This work was carried out under the auspices of the Birmingham Branch of the British Empire Cancer Campaign.

### *Estimation of Free Adenine*

Add 0.10 g. zinc dust to 20–30 ml. adenine solution containing 5–40  $\mu\text{g.}/\text{ml.}$  in 1  $N$   $\text{H}_2\text{SO}_4$ . Stand in a water-bath at  $90^\circ\text{C.}$  for 30 min. with an occasional gentle swirl, cool, and filter through a very small pad of cotton wool. Remove samples, e.g., 2 ml. to graduated stoppered cylinders. Add 1.0 ml. of 0.1%  $\text{NaNO}_3$  mix, and after standing for 10 min. add 1.0 ml. of 0.1% ammonium sulfamate and mix thoroughly; after 2 min. add 1.0 ml. freshly made Bratton-Marshall reagent. Make up the volume to 10 ml. with distilled water and measure the color after 10 min. When estimated on the Spekker Absorptiometer with an Ilford color filter H603 the color intensity follows Beer's Law with amounts of adenine within the above limits. The color value of subsequent solutions can be read from the linear curve. Blank tests should give no discernible color. It was confirmed that of the purines and pyrimidines occurring in nucleic acids, adenine alone gives a colored product and the presence of the other bases does not affect the estimation.

### *Estimation in Nucleotides and Nucleic Acids, etc.*

By hydrolysis with mineral acid the adenine in these compounds may be liberated without its decomposition; refluxing for 1 hr. with 1  $N$   $\text{H}_2\text{SO}_4$  was satisfactory, as shown by the following experiments.

#### *1. Estimation in adenosine.*

Ten-ml. samples of adenosine solution (30 mg./100 ml.) were refluxed with 4 ml. 4  $N$   $\text{H}_2\text{SO}_4$  and 2 ml. water for 1 hr. The solution after cooling was made up to 40 ml. Ten-ml. portions of this solution were acidified with 20 ml. 2.5  $N$   $\text{H}_2\text{SO}_4$  and reduced with zinc as described above.

Four ml. of this solution was found to contain 48.8  $\mu\text{g.}$  adenine. (This value is the mean of two determinations on each of four reduced solutions.  $\bar{W} = 1.4 \mu\text{g.}$ )

Theoretical value for pure adenosine of similar concentration is 50.0  $\mu\text{g.}$

It is evident that products derived from the hydrolysis of the pentose do not interfere with the determination.

#### *2. Estimation in adenylic acid (Yeast adenylic acid; adenosine-3-phosphoric acid).*

Ten-ml. portions of solution containing 4 mg. adenylic acid were hydrolyzed with 10 ml. 2  $N$   $\text{H}_2\text{SO}_4$  for 1 hr. and diluted to 40 ml. Ten ml. with 10 ml. distilled water and 10 ml. 5  $N$   $\text{H}_2\text{SO}_4$  were reduced with zinc for 30 min. Four-ml. samples were submitted to color development.

Adenine found per 4-ml. aliquot = 51  $\mu\text{g.}$  (Average of 8 determinations.)

Theoretical adenine content of pure acid = 52.6  $\mu\text{g.}$

#### *3. Estimation in commercial thymus nucleic acid (sodium salt).*

The solution contained 0.20% of the preparation.

Ten ml. was hydrolyzed and diluted to 40 ml. from which 10-ml. portions were reduced with zinc in the usual way. Four-ml. samples of the reduced solution were used for estimation.

Adenine found = 42.5  $\mu\text{g.}$  (Average of 8 determinations.) The adenine present in a solution of similar concentration of pure nucleic acid would be 63.5  $\mu\text{g.}$  assuming a tetranucleotide formula with 1 unit of adenine.

The specimen of nucleic acid was found to contain 7.4% phosphorus suggesting

that it was approximately 76% pure. Thus the samples of hydrolyzates analyzed would be expected to contain 48  $\mu$ g. adenine. It may be concluded that either the recovery was not quite quantitative or that actually a smaller molar porportion of this unit is present in the polynucleotide than would be expected on the tetranucleotide formula.

4. *Estimation in yeast nucleic acid (sodium salt).*

Ten-ml. samples from a sample containing 20 mg.-% of commercial material were hydrolyzed and submitted to the reduction process. Four ml. of hydrolyzed, reduced solution (containing 0.667 mg. nucleic acid) was found to give a color value equivalent to 45  $\mu$ g. adenine.

Phosphorus content of nucleic acid = 7.4%.

Theoretical content assuming 4 phosphoric acid radicals in a mole of weight 1390 = 9.5%, i.e., apparent purity on P content.

On a similar basis the theoretical adenine in the above (= 76%) aliquots would be 65  $\mu$ g.; i.e., apparent purity by adenine content = 69%.

### SUMMARY

Adenine may be estimated after reduction with zinc dust in acid solution by diazotizing and coupling with *N*-(1-naphthyl) ethylene-diamine hydrochloride.

Quantities of 10–80  $\mu$ g. can be estimated on the Spekker Absorptiometer with 5% accuracy utilizing a final volume of 10 ml.

Adenine in nucleoside or nucleotide combinations can be liberated by hydrolysis and estimated satisfactorily.

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# The Quantities of Amino Acids in the Nonprotein Fraction of Breast and Cow's Milk

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## INTRODUCTION

The essential amino acid composition of several samples of cow and breast milk were reported by Block and Bolling (1). Their results, in agreement with the literature [cf. (1)], indicated little or no difference between the amino acid composition of human and cow's milks. This is the case even though the content of certain amino acids, especially the sulfur amino acids, is higher in cow's lactalbumin than in casein and the fact that breast milk contains relatively more lactalbumin than cow's milk. In spite of these differences in protein composition, the amino acid patterns in the total proteins of the two milks do not show significant differences. These results are in agreement with the findings of Williamson (2) who states that when cow's milk is diluted with water so that the total protein is similar to that of pooled breast milk, there is no significant difference in the molar concentration of the sum of sulfur amino acids of the two types of milk. Furthermore, according to Williamson (2) diluted cow's milk contains higher percentages of valine, threonine, and histidine while breast milk was richer in tryptophan. Recent clinical (3, 4) results are also in agreement with the suggestion that breast milk is not better than cow's milk for the protein nutrition of the human infant. Thus Gordon *et al.* (3) report that greater weight gains of premature babies were achieved when they were fed partially skimmed milk or evaporated milk than when breast milk was used. Albanese *et al.* (4) are also on record concerning the desirability of feeding to infants cow's milk; they say, "On the basis of calculations from the available data on the sulfur amino acid content of human milk, it would appear that breast milk provides a very

meagre margin of safety during the early months of life and that breast milk does not provide an adequate quantity of the sulfur amino acids in the latter part of the first year. The margin of safety is considerably greater in the artificially fed infant."

Thus, the often expressed opinion that breast milk is superior for the *protein* nutrition of the human infant must be based either on differences in the nonprotein amino acid fraction or on teleological reasoning. The results reported in this paper indicate that the essential amino acid pattern in the nonprotein fractions of breast milk are not significantly different from those of cow's milk.

## EXPERIMENTAL

### *Preparation of Protein-Free Filtrates*

*From Breast Milk and Human Colostrum.* 2.15 l. of mature milk (3 months or longer post partum) were heated with stirring to boiling and the pH adjusted with  $\text{H}_3\text{PO}_4$  to 4.55. After standing over night the precipitate was removed by filtration. This filtrate still contained protein as indicated by a  $\text{CCl}_3\text{COOH}$  test. The protein was then removed by precipitation with ammonium sulfate at pH 4. The resulting precipitate was discarded. The  $\text{NH}_3$  and  $\text{SO}_4^{=}$  were removed with  $\text{Ca}(\text{OH})_2$  in the usual manner. The resulting solution contained only a trace of ammonia. The amino acids and peptides were adsorbed on a cation exchange column (Duolite C-3) in the hydrogen cycle. The column was washed until a sample of the washings gave a negative test for carbohydrate. The amino acids and peptides were then eluted with 7% of aqueous  $\text{NH}_3$  until an aliquot of the elutriate gave a negative test with ninhydrin. The ammonia elutriate was concentrated to a small volume to remove the excess  $\text{NH}_3$  and the solution was ready for amino acid analysis. It should be noted that this process separates nonadsorbable nitrogenous substances as well as lactose from the amino acids and peptides.

*From Cow's Milk.* Dry-skimmed and evaporated-skimmed milk were used in these experiments. The proteins were apparently completely precipitated by first adjusting the reconstituted milk to pH 4.55 at 55°C. to remove the casein and then to pH 6.0 with NaOH to coagulate the lactalbumin at 95–9°C. for 30 min. The separation of the amino acids and peptides from carbohydrates and nonadsorbable nitrogenous constituents was carried out on Duolite C-3 as described above.

*Analytical Methods*

The same analytical procedures for nitrogen and the amino acids were used as described in an earlier publication (1). The amino nitrogen was estimated by the copper precipitation method of Pope and Stevens [cf. (5)].

## RESULTS AND SUMMARY

The results in Table I show the approximate amino acid distribution in the protein [taken from (1)] and the nonprotein fractions of cow's milk and of human milk and colostrum. Although breast milk contains

TABLE I

*Approximate Quantities of Amino Acids in Nonprotein and Protein Fractions of Breast and Cow's Milk*

Calculated in grams of amino acid/16.0 g. of N for the proteins, and in grams of amino acid/16.0 g. of amino N for the nonprotein fractions

Amino acid	Mature breast milk <sup>a</sup>		Human colostrum		Cow's milk <sup>c</sup> (Dry skim)	
	N. P. fraction	Proteins <sup>b</sup> (1)	N. P. fraction	Proteins (1)	N. P. fraction	Proteins (1)
Lysine	6.8 <sup>b</sup>	7.2 <sup>b</sup>	7.9 <sup>b</sup>	6.5	9.8 <sup>b</sup>	8.2 <sup>b</sup>
Tyrosine	3.5	5.2	6.6	5.4	2.6	6.2
Tryptophan	0.5	1.6	1.0	2.0	1.2	1.7
Cystine	1.4	1.6	1.8	2.5	2.2	0.8
Methionine	3.0	2.2		1.8	3.6	3.4
Threonine	4.0	4.5	5.7	5.0	3.8	4.3
Leucine	6.3 <sup>b</sup>	9.8 <sup>b</sup>	6.2 <sup>b</sup>	7.9 <sup>b</sup>	8.0 <sup>b</sup>	8.7 <sup>b</sup>
Isoleucine	5.9 <sup>b</sup>	7.5 <sup>c</sup>	4.1 <sup>b</sup>	5.4 <sup>b</sup>	6.2 <sup>b</sup>	7.0 <sup>b</sup>
Valine	6.5 <sup>b</sup>	8.8 <sup>b</sup>	6.2 <sup>b</sup>	6.9 <sup>b</sup>	7.8 <sup>b</sup>	6.5 <sup>b</sup>
Amino N <sup>d</sup> /total N.P.N.	78%		68%		50%	

<sup>a</sup> Breast milk contained 18.5% of nonprotein N (N.P.N.); cow's milk contained 5.3% of N.P.N. and human colostrum contained 22.3% of N.P.N.

<sup>b</sup> Microbiological methods.

<sup>c</sup> The milk proteins from one woman (White A) reported previously (1) contained 5.1 g. of tyrosine, 3.2 g. of tryptophan, 4.0 g. of cystine, and 2.5 g. of methionine per 16.0 g. of nitrogen. Since that time, she has given birth to another child. The proteins prepared from her milk, collected 3 months post partum, yielded 5.3 g. of tyrosine, 2.6 g. of tryptophan, 3.3 g. of cystine, and 2.3 g. of methionine per 16.0 g. of nitrogen. The air-dried proteins contained 15.1% of nitrogen, uncorrected for volatile matter and ash.

<sup>d</sup> Amino nitrogen of the N.P.N. fraction after acid hydrolysis.

TABLE II

*Approximate Quantities of Amino Acids in Nonprotein and Protein Fractions of Breast and Cow's Milk*

Calculated in milligrams of amino acid/100 ml. of milk or colostrum

Amino acid	Mature breast milk		Human colostrum		Cow's milk	
	N. P. fraction	Proteins	N. P. fraction	Proteins	N. P. fraction	Protein-
Lysine	10	101	21	130	10	328
Tyrosine	5	73	17	108	3	248
Tryptophan	1	22	3	40	1	68
Cystine	2	22	5	50	2	32
Methionine	5	41		36	4	136
Threonine	6	63	15	100	4	172
Leucine	9	137	16	158	9	348
Isoleucine	9	105	11	108	7	280
Valine	10	123	16	138	8	260

approximately four times as much nonprotein nitrogen as does cow's milk, the amino acid patterns of the protein and the nonprotein fractions of breast milk and of cow's milk are essentially similar. The data in Table I, calculated in terms of milligrams of amino acid/100 ml. of milk or colostrum, are presented in Table II in order to facilitate their use by nutritionists.

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# Biophysical Studies of Blood Plasma Proteins. XIV. Separation of Gamma Globulins from Normal Hog Serum <sup>1</sup>

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## INTRODUCTION

More or less specific techniques involving the use of ethanol as a fractionating agent have been developed for the rapid and efficient removal of the antibody-rich proteins from various animal sera. To date these methods have been applied to human, rabbit, dog, goat, guinea pig, chicken, rat (1), horse (2), and bovine (3, 4) sera. In view of the ready availability of hog serum, it seemed desirable to extend the work to include this material and thus make available another antibody system to which immunochemical techniques may be applied. In the present investigation specific fractionation conditions have been developed for the separation of the hog serum  $\gamma$ -globulins. In addition, certain physical properties of these proteins have been studied.

## EXPERIMENTAL

Pooled normal hog serum served as the source of starting material. The usual precautions employed in alcohol fractionations, *i.e.*, rigid control of pH, ionic strength, temperature, alcohol concentration, protein concentration, etc. (5) were observed. The progress of the fractionation was followed by means of routine electrophoretic analyses, carried out on 2% protein solutions in pH 8.6 barbiturate buffer of ionic strength 0.1, at a constant potential gradient of approximately 6.2 volts/cm. The duration of the experiments was 3 hr. The electrophoretic patterns obtained under these conditions were used to evaluate yield and purity of product.

Additional electrophoretic experiments were performed to provide mobility data. Such experiments were carried out under conditions similar to those described above except that the buffer used in the experiments at ionic strength, 0.1, consisted of 0.02

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<sup>1</sup> This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

$M$  buffer salt and  $0.08 M$  NaCl, while in the experiments at ionic strength,  $0.01$ , buffer salt alone was used. Heterogeneity constants were obtained from the electrophoretic patterns of experiments performed at the isoelectric point in  $0.1$  ionic strength buffers, using the method of Alberty (6).

The sedimentation analyses were performed in a Svedberg oil turbine ultracentrifuge operated at  $185,000 \times g$ . A Schlieren optical method was used to record the position of the boundary as a function of time.

Diffusion studies were carried out at approximately  $1^\circ\text{C}$ . in the electrophoresis apparatus and the values of the diffusion constant corrected to water at  $20^\circ\text{C}$ . In the experimental procedure, the boundary-sharpening technique of Kahn and Polson (7) and the rectangular slit advocated by Anderson and Alberty (8) were employed.

## RESULTS

As a result of a number of fractionation experiments with hog serum for the purpose of recovering  $\gamma$ -globulin, the procedure shown in Diagram 1 was evolved. Included in the outline are yield and purity of protein data expressed in the conventional way. Descending electrophoretic patterns for the several protein products obtained at various steps in the fractionation are presented in Fig. 1.

The experimental conditions used at the first stage to separate the  $\gamma$ -globulins in crude form are consistent with those previously reported for other animal serums (1, 2, 3). The conditions required to remove  $\beta$ -globulins from the antibody-rich fraction, Ppt. A, while maintaining the  $\gamma$ -globulins in solution were subjected to detailed and careful study. Inspection of the electrophoretic diagrams of Fig. 1 shows that reason-

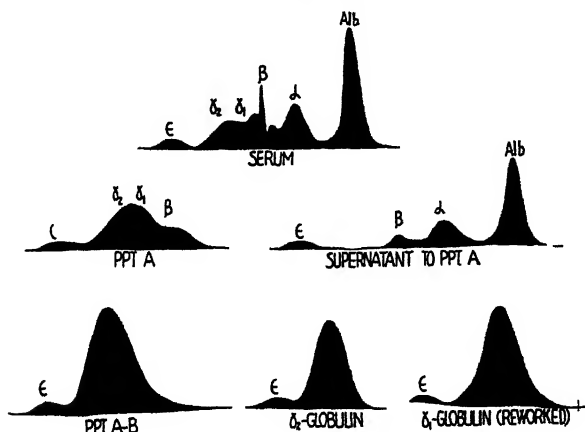


FIG. 1. Electrophoretic diagrams of hog serum and fractions.

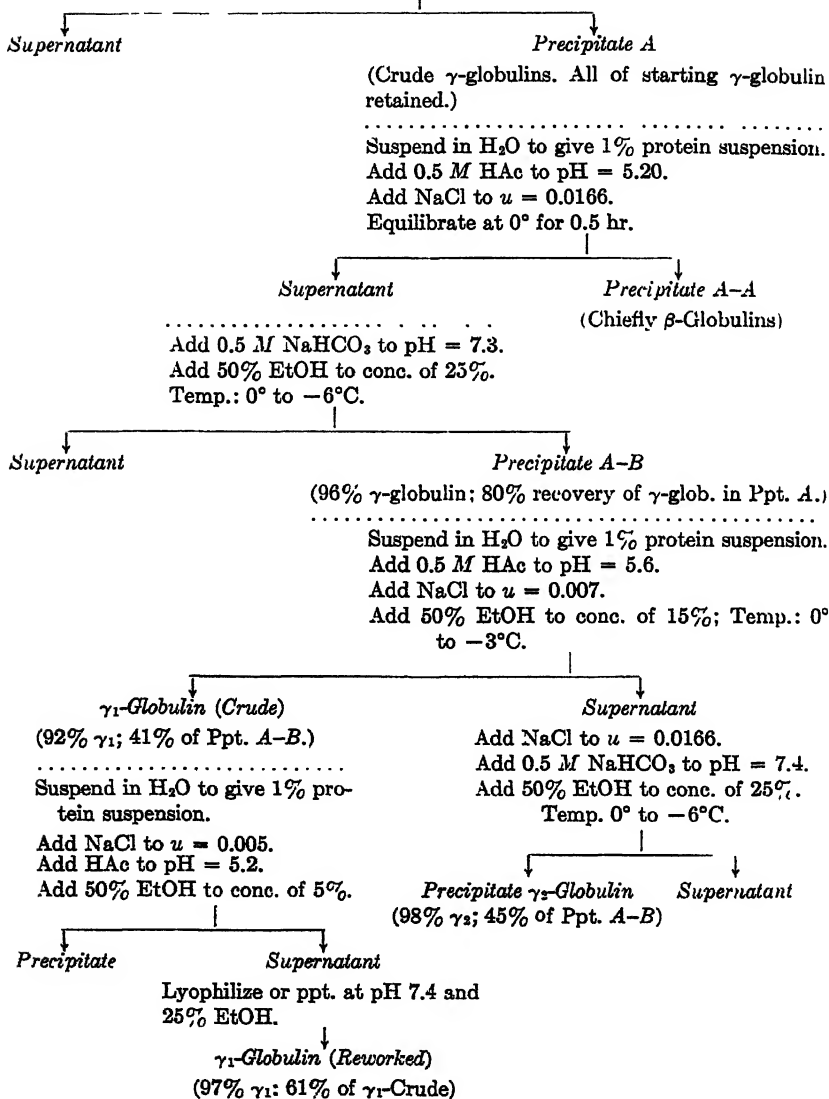
DIAGRAM 1. *Schematic Fractionation Procedure for Hog Serum*

1 volume serum diluted with 3 vol. H<sub>2</sub>O.

Add 0.5 M NaHCO<sub>3</sub> to pH = 7.8.

Add 50% EtOH to conc. of 20%.

Temp.: 0° to -6°C.



able success has been obtained in the rework of Ppt. A to give a Ppt. A-B, which contains little  $\beta$ -globulin. Precipitate A-B, whose electrophoretic composition is shown in Fig. 1, was subjected to further sub-fractionation to give a  $\gamma_2$ -globulin and a  $\gamma_1$ -globulin fraction. It should be remarked, however, that we were never able to remove completely the  $\beta$ -globulin from the protein we have described as Ppt. A-B. The recovery of  $\gamma$ -globulins is based on the assumption that the serum antibody fraction is made up of equal amounts of  $\gamma_1$ - and  $\gamma_2$ -globulins. The hog serum used in these studies contained from 6.8 to 7.5% protein of which approximately 25% is  $\gamma$ -globulin. Following through the various rework steps we found that the over-all yield of the  $\gamma_2$ -globulin was some 72% while that of the  $\gamma_1$ -globulin rework amounted to 40%. A higher yield of the proteins comprising these two fractions may be obtained if one utilizes Ppt. A-B (see Diagram 1).

The electrophoretic mobilities of the  $\gamma_2$ - and  $\gamma_1$ -globulin fractions at pH 8.6 are  $-1.7$  and  $-2.5 \times 10^{-5}$  cm.<sup>2</sup>-volt<sup>-1</sup>-sec.<sup>-1</sup>, respectively. Casual inspection of the patterns, taken on the descending side in each case, suggests that the  $\gamma_2$ - and  $\gamma_1$ -globulins are what have been termed "pure" in the literature. However, if one studies the outline of these curves in a quantitative fashion by the method of Alberty (6), heterogeneity constants ( $H$ ), of  $0.74$  and  $0.87 \times 10^{-5}$  cm.<sup>2</sup>-volt<sup>-1</sup>-sec.<sup>-1</sup>, respectively, are obtained for the  $\gamma_2$ - and  $\gamma_1$ -globulin fractions. These values have been obtained from a study of the electrophoretic spreading of these proteins, the data of which are plotted to give Fig. 2.

Within experimental error, the  $\gamma_1$ -globulin and the  $\gamma_2$ -globulin fractions have the same sedimentation constant,  $s_{20w} = 7.1 S$  at a protein concentration of 1% in 0.15 *M* NaCl. This figure may be compared to the value of  $s_{20w} = 6.8 S$  found by Kabat (9) for one of the hog antibody components which he obtained, and the figure of 6.9 found by Koenig (10) for a 1% solution of hog  $\gamma$ -globulin (Fraction II). It is also comparable in magnitude to the sedimentation constants which are usually reported for the major component of most animal  $\gamma$ -globulins. It might be remarked that we did not find any component of sedimentation constant  $s_{20w} = 18 S$ , although protein corresponding to this sedimentation constant made up the largest percentage of Kabat's hog pneumococcus antibody preparation. Within the limits of accuracy of the method used, the sedimentation constant shows no detectable dependency on concentration in the region 0.3-1.2% protein. In sedimentation studies of hog  $\gamma$ -globulin (Fraction II), Koenig

(10) likewise found little concentration dependency over this protein range. The  $\gamma_1$ -globulin possessed a small amount, approximately 3%, of a component with somewhat higher sedimentation constant as indicated by a somewhat asymmetrical sedimentation pattern. A similar component has been reported (2, 12-15) for many  $\gamma$ -globulin preparations. The  $\gamma_2$ -globulin appeared to be homogeneous as regards its mass properties.

The average value of the diffusion constant obtained for the  $\gamma_1$ - and  $\gamma_2$ -globulin fractions was  $D_{20w} = 3.85 \times 10^{-7}$  cm.<sup>2</sup>sec.<sup>-1</sup>. A close approximation of this value is also obtained from the plot of apparent diffusion constant against time (see Fig. 2).

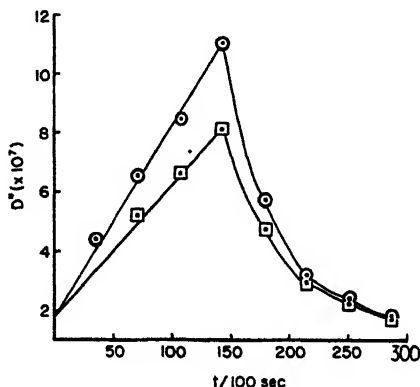


Fig. 2. Electrophoretic spreading experiments for hog  $\gamma$ -globulins in 0.1 ionic strength buffers at their average isoelectric point ○ =  $\gamma_1$ -globulins; □ =  $\gamma_2$ -globulins. The average values of the apparent diffusion constant for the two limbs are given.

It is interesting that the  $\gamma_1$ -globulin and the  $\gamma_2$ -globulin fractions have the same sedimentation and diffusion constants. Assuming the apparent specific volume of the globulins to be the same as that reported by Kabat (9) for hog antipneumococcus globulin, 0.715, and using the sedimentation and diffusion data we have obtained, a molecular weight of 168,000 is calculated. The molecular weights of various normal and immune globulins which have been reported in the literature are usually very close to this figure.

Electrophoretic mobility data are required to prove that the  $\gamma_1$ - and  $\gamma_2$ -globulin protein fractions are essentially different. In Fig. 3 are presented such data for these two hog  $\gamma$ -globulin preparations at vari-

ous pH values. It will be seen that the usual plot of mobility *vs.* pH gives the isoelectric point of  $\gamma_1$ -globulin at pH 6.1 while that of  $\gamma_2$ -globulin is very close to 7.2. Only a slight difference in the isoelectric point of  $\gamma_2$ -globulin in ionic strength buffers of 0.1 and 0.01 is indicated. These values are seen to differ considerably from the isoelectric point value, pH = 5.1, reported by Tiselius and Kabat (16) for an antibody preparation obtained by the dissociation of the antigen-antibody complex.

It is unusual that the isoelectric point of the  $\gamma_2$ -globulin is relatively insensitive to variation in ionic strength. Furthermore, the mobility curves for the protein at 0.1 and 0.01 ionic strength are appreciably

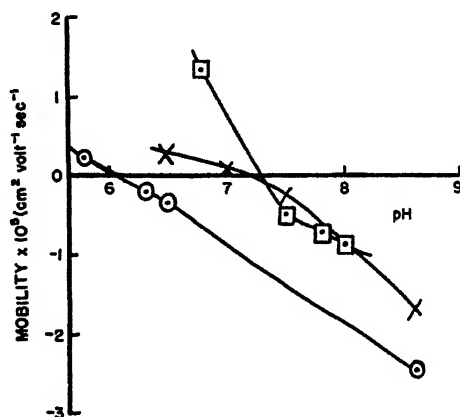


FIG. 3. Electrophoretic mobilities of hog  $\gamma$ -globulin at various pH values.  $\bigcirc$  =  $\gamma_1$ -globulin at ionic strength 0.1,  $\times$  =  $\gamma_2$ -globulin at ionic strength 0.1,  $\square$  =  $\gamma_2$ -globulin at ionic strength 0.01.

different in form. One is concave upward, the other concave downward. A similar phenomenon is shown by bovine  $\gamma_2$ -globulin in the region of its isoelectric point (4). Ordinarily, these curves are somewhat parallel and the isoelectric point increases with decreasing ionic strength. It was found impossible to determine the isoelectric point of the  $\gamma_1$ -globulin fraction at ionic strength, 0.01, due to the insolubility of the material under this condition. The difference in solubility is an additional indication of a difference between the two  $\gamma$ -globulin fractions.

Using the values of the slope of the mobility-pH curves and the heterogeneity constant, the isoelectric point distribution for each of

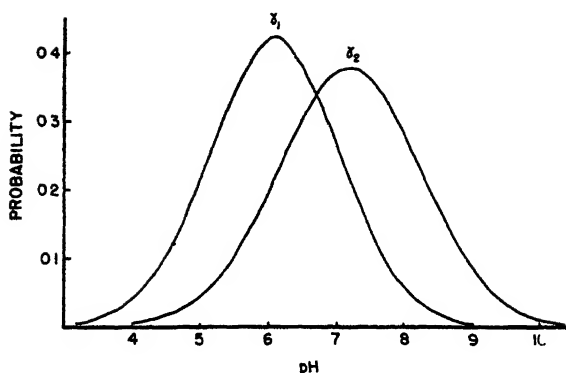


FIG. 4. Isoelectric point distributions for porcine  $\gamma_1$ - and  $\gamma_2$ -globulins at 0.1 ionic strength.

the two globulin fractions was calculated. Plots of these data, shown in Fig. 4, reveal marked overlap of the isoelectric point distributions of the proteins. In general, the physical properties of the two porcine  $\gamma$ -globulins appear to resemble most closely the corresponding bovine globulins. Using the electrophoretic mobility data of Hess and Deutsch (4), the isoelectric point distributions for bovine  $\gamma_1$ - and  $\gamma_2$ -globulins have been computed for ionic strength, 0.1. These data are presented in Fig. 5. The overlap of isoelectric point in this case is appreciably less as com-

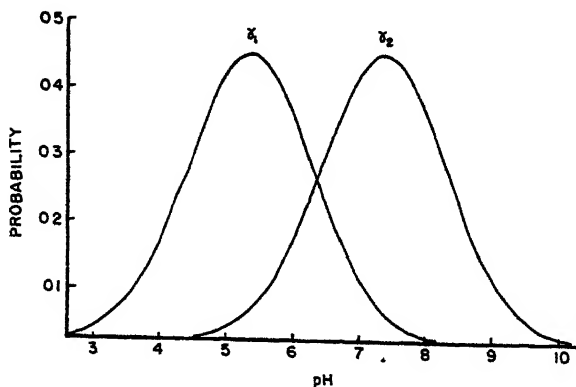


FIG. 5. Isoelectric point distribution for bovine  $\gamma_1$ - and  $\gamma_2$ -globulins at 0.1 ionic strength.

pared to that found in the case of the analogous porcine  $\gamma$ -globulins, indicating that the bovine globulin system lends itself somewhat better to chemical separation into two fractions. Comparison of the above with the data of Alberty (17) for two human serum  $\gamma$ -globulin fractions indicates that both the porcine and bovine  $\gamma$ -globulins show considerably more overlap than do the corresponding globulins of human serum.

The crude  $\gamma$ -globulin fraction (Ppt. A of Diagram 1) has been found to possess the antibody activity of hog anti-cholera serum. Thus this precipitate possessed the comparable protective action of the original serum while the supernatant proteins to Ppt. A had no demonstrable activity.<sup>2</sup>

#### ACKNOWLEDGMENTS

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#### SUMMARY

A chemical fractionation method for the recovery, and subsequent separation into two fractions, of the  $\gamma$ -globulin constituents of porcine serum has been presented. The two  $\gamma$ -globulins have been characterized by sedimentation, diffusion, and electrophoretic analyses.

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# Destruction of Influenza A Virus Infectivity by Amino Compounds<sup>1</sup>

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## INTRODUCTION

The destruction of the infectivity of PR8 influenza A virus has been under study in the author's laboratory. The effects of heat (1), pH (1), urea (2), and formaldehyde (3) have been reported thus far. It was thought worthwhile to investigate the action of certain amino compounds on the infectivity of this virus.

Nucleic acid has been reported on several occasions to counteract the effect of biochemically active compounds containing amino groups. For example, Fitzgerald and Lee (4) showed that ribose nucleic acid was able to counteract the inhibitory effect of various acridines on the lysis of *E. coli* by bacteriophage. Similarly, Bichowsky-Slomnitzki (5) observed that nucleic acid suppressed the antibacterial activity of stilbamidine and pentamidine. In view of the foregoing, experiments were also carried out on the protection of influenza virus from the action of amino compounds by nucleic acid.

## MATERIALS

Four different preparations of PR8 influenza A virus, each of which consisted of unmodified allantoic fluid from diseased chicken embryos, were used throughout these investigations. Preparation 1 was the same as Prepn. D described by Lauffer, Carnelly, and MacDonald (1). Preparations 2, 3, and 4 were the same as Prepn. B, C, and D, respectively, described by Lauffer and Wheatley (3). The yeast nucleic acid was obtained from Schwarz Laboratories, lot HN 4705.

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## METHODS

Inactivation experiments were carried out in the following manner. The virus preparation was diluted, usually 100 or 1000-fold, with 0.1 *M* potassium phosphate buffer at pH 7. Small amounts of the amino compound were dissolved in water, 0.1 *M* phosphate buffer, or 1 *M* phosphate buffer at pH 7, depending upon the degree of basicity of the amine. Equal quantities of the diluted virus and the diluted amine were mixed, and the resultant solution was held at some fixed temperature for a specified period of time. At the end of this time, the mixture was diluted, and 50% chicken embryo infectivity endpoints were determined in the manner described previously (1). Similar virus activity titrations were carried out on the untreated virus sample. In some experiments, neutralized nucleic acid solutions were also mixed with the virus and the amine solutions.

## RESULTS

The results obtained when aniline was added to influenza virus under a variety of conditions are shown in Table I. Virus Prepn. 1 was used in obtaining all of the data presented in this table. The titer of the untreated virus was determined in six separate experiments and was found to have a mean value of 8.4. The effect of the aniline for each condition of treatment can be judged by the difference between the titers listed in the extreme right hand column of Table I and this mean titer for the untreated virus.

Some of the data in Table I show the relationship between the infectivity titer and the time of reaction between virus and aniline under otherwise fixed conditions. It can be shown by plotting infectivity titers against time of treatment that the data do not fit any simple kinetic process. The equation of a first-order reaction can be fitted to the data representing the initial stages, but the survival after long times of reaction is too great to fit the first-order law.

By comparing these data with those published previously by Lauffer, Carnelly, and MacDonald (1) for the inactivation of influenza virus in the absence of added chemical agents, it can be seen that, in the presence of 0.05% aniline, the inactivation proceeds about as fast at 35°C. as it does at 45°C. in the absence of aniline. In the presence of 0.5% aniline, the reaction proceeds at about this same rate at 24.5°C. Also, in the presence of 0.5% aniline, the reaction proceeds about as fast at 35°C. as at 55°C. in the absence of added chemicals.

The data of Table I also indicate that the extent of inactivation depends upon the initial virus concentration. In experiments carried out with 0.5% aniline in contact with virus for 15 min. at 24.5°C., the loss of virus activity was about 100-fold for the virus at a dilution of one-

TABLE I

*The Effect of Aniline on the Infectivity of PR8 Influenza A virus*

Virus conc. (vol.-% of stock virus) (i.e., 10 <sup>-3</sup> virus = 0.1%)	Aniline conc. (wt.-%)	Temp. (°C.)	Time (min.)	Titer <sup>a</sup>
30	0.5	24.5	15	7.1
5	0.5	24.5	15	6.8
0.5	0.5	24.5	5	6.8
0.5	0.5	24.5	5	6.0
0.5	0.5	24.5	10	6.6
0.5	0.5	24.5	15	5.8
0.5	0.5	24.5	15	6.6
0.5	0.5	24.5	20	6.0
0.5	0.5	24.5	30	6.0
0.5	0.5	24.5	30	5.5
0.5	0.5	24.5	60	4.4
0.5	0.5	24.5	120	3.6
0.05	0.5	24.5	15	5.6
0.005	0.5	24.5	5	6.0
0.5	0.5	24.5	15	5.6
0.5	0.5	35	3	6.0
0.5	0.5	35	5	4.6
0.5	0.5	35	10	4.3
0.5	0.05	35	5	7.7
0.5	0.05	35	10	6.7
0.5	0.05	35	15	6.0
0.5	0.05	35	30	6.3
0.5	0.05	35	60	3.4

<sup>a</sup> Titer is expressed as the negative logarithm of that concentration of virus containing allantoic fluid in diluent which will produce infection in 50% of chicken embryos inoculated with 0.2 ml. each. Titer of untreated virus (control) was 8.4, the mean of 6 determinations (1).

half, about 1000-fold for the virus at a dilution of 1/20, and about 10,000-fold for the virus at dilutions of 1/200, 1/2000, and 1/20,000.

Studies on the effect of methylaniline and dimethylaniline on influenza virus activity were carried out with Virus Prepn. 4. When a

system containing 0.5% methylaniline and virus at a concentration of  $0.5 \times 10^{-3}$ , buffered with 0.1 M phosphate buffer at pH 7, was held for 10 min. at room temperature, the infectivity titer was reduced from a value of 7.5 for the control to 4.1. When an experiment was carried out with dimethylaniline under exactly the same conditions, the titer was reduced only to 6.6. Thus, it can be seen that methylaniline inactivated the virus to about the same extent as aniline itself, but dimethylaniline had much less, if any, effect. The interpretation of this result is obscured somewhat by virtue of the fact that methyl- and dimethylaniline formed unstable emulsions, while the aniline dissolved completely.

TABLE II

*The Effect in the Presence and in the Absence of Nucleic Acid of Aminoethanol on the Infectivity of PR8 Influenza A virus*

Test no	Virus conc. (vol.-% of stock)	Aminoethanol (wt.-%)	Nucleic acid (wt.-%)	Titer	Control	Prep.
1	0.05	0.5	0	5.6	8.1	C
2	0.03	0.3	3.3	7.1	8.1	C
3	0.05	0.5	0	6.0	6.8	C
4	0.03	0.3	3.3	6.8	6.8	C
5	0.05	0.5	0	5.3	7.5	D
6	0.03	0.3	3.3	6.5	7.5	D
7	0.5	0.5	0	5.9	8.0	B
8	0.05	0.05	0	3.8	7.3	B

Two kinds of experiments were carried out involving the action of aminoethanol on influenza virus. Since aminoethanol is a relatively strong base, it was necessary to use molar phosphate buffer at pH 7 as a diluent. By this means, the pH of the final virus aminoethanol mixtures could be kept to within half a unit of neutrality. In the first kind of experiment, virus and aminoethanol were mixed to give the final concentrations shown for tests 1, 3, 5, 7, and 8 in Table II, and the mixtures were allowed to stand at room temperature for 10 min. Infectivity titers of untreated and treated virus were then measured and are recorded in Table II. It can be seen that aminoethanol, when the concentration is either 0.5% or 0.05%, caused substantial decreases in virus titer.

Experiments of the second sort were carried out in which the aminoethanol was first mixed with nucleic acid solution and allowed to stand 10 min. at room temperature, and then virus solution was added to give the final concentrations of virus, aminoethanol, and nucleic acid shown for tests 2, 4, and 6 in Table II. After 10 min. at room temperature, the infectivity titers were determined and compared with those of the controls. Test 1 was carried out at the same time as test 2, the only difference being that no nucleic acid was added in test 1. Similar relationships hold between tests 3 and 4 and between tests 5 and 6.

The data show that the virus infectivity titers were reduced less in the presence of both nucleic acid and aminoethanol than when the virus was subjected to aminoethanol in the absence of nucleic acid. The mean difference for corresponding tests between the titers in the presence of nucleic acid and in the absence of nucleic acid is 1.2. Lauffer, Carnelly, and MacDonald (1) obtained a value of 0.35 for the standard deviation of the distribution of titers when 5 embryos were used per dilution. Knight (6) obtained a value of about 0.23 when 5 embryos were used per dilution. Thus, it can be considered that a safe estimate of the standard deviation of the distribution of titers is 0.35 units. The standard error of the difference between two consecutive titers should be equal to  $\sqrt{2} \times 0.35$  or 0.50. The standard error for the mean of three consecutive differences should be  $0.50/\sqrt{3}$  or 0.29. The mean difference observed, 1.2, is 4.1 times the standard error of mean differences, 0.29. It seems, therefore, safe to conclude that the addition of the nucleic acid preparation caused a real decrease in the effectiveness of the aminoethanol upon influenza virus.

### DISCUSSION

The effect of any chemical agent upon virus infectivity could be due, in principle, to an effect upon the host, to an effect upon the virus, or to both. If the effect is upon the virus, it could be the result of either the reversible formation of an inactive complex between virus and agent or of an irreversible destruction of biological activity analogous to that which occurs when virus is subjected to high temperatures. The fact that the extent of the decrease in infectivity titer depends upon the conditions under which virus and aniline or other amines are held together prior to introduction into the chicken embryos shows that, in this case, the effect of the agent is upon the virus. It does not rule out

the possibility that there may also be an effect upon the host. The fact that, in the case of aniline, the extent of the effect was shown to increase with the time of contact eliminates the possibility of an essentially instantaneous formation of an inactive equilibrium complex between virus and aniline. The fact that the extent of the inactivation is greater when the virus and aniline are held at 35°C. than when held at 24.5°C. also indicates that the limiting factor is the rate at which virus is destroyed in the presence of aniline and not the extent to which an equilibrium complex is formed.

### SUMMARY

1. Neutral solutions containing 0.5% or less of aniline greatly accelerated the destruction of PR8 influenza A virus infectivity.
2. The destruction did not follow any simple kinetic pattern.
3. Methylaniline also inactivated the virus, but dimethylaniline had a less pronounced effect.
4. Neutral solutions containing 0.5% or less of aminoethanol also caused the destruction of virus infectivity at room temperature.
5. A neutralized yeast nucleic acid preparation at a concentration of 3.3% reduced substantially the effect of the aminoethanol.

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# The Effect of Precipitation and Dehydration on the Physiologic and Enzymatic Properties of Myosin and Actomyosin

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## INTRODUCTION

From the finding of Engelhardt and Liubimova that myosin preparations contain adenosine triphosphatase (ATP-ase) activity (1) the question arose as to the identity of myosin and the phosphate-splitting enzyme. Reports from various laboratories lend support to the concept that the phosphate-splitting enzyme may constitute only a fraction of the myosin molecule or an adsorbed component on the parent myosin structure (2-4). Singher and Meister, for example, observed that myosin B dialyzed against pH 6 buffer lost ATP-ase activity with no corresponding decrease in double refraction of flow (2). The objective of this study was to obtain a myosin either physiologically intact and enzymatically inactive or the converse thereof. An approach to the problem was suggested by the notation of Straub and associates that the muscle protein actin is stable on precipitation at the isoelectric point in the absence of salt (5). If dialyzed myosin were likewise more stable it might conceivably submit to more rigorous separation procedures. It was also considered that myosin in the presence of actin may retain physiological properties to a different extent than enzymatic activity after isoelectric precipitation or after dehydration.

## EXPERIMENTAL

As an indication of the integrity of the myosin structure, the superprecipitation reaction of actomyosin was employed (6). This test is carried out by adding myosin and actin in a ratio of 2.5:1 in a tube containing buffer of pH 7.0 and water to bring the salt concentration

to approximately 0.1 *M*. The precipitate resulting from addition of a drop of adenosine triphosphate (ATP) solution viewed in proper lighting may be judged according to density, texture, contractility, and time of appearance.

Enzymatic activity was determined by measuring the increase in inorganic phosphate during incubation of myosin and ATP, using the Fiske-SubbaRow method for inorganic phosphate (7). The myosin was incubated 5 min. at 37° in an excess of ATP and in presence of 0.5 *M* KCl, after which the reaction was stopped with 0.5 ml. of 20% trichloroacetic acid. In order to obviate heavy-metal inhibition of myosin, water distilled from glass vessels was used exclusively and, as a further precaution, glycine was added at a concentration of 0.1 *M* in the enzyme test. CaCl<sub>2</sub> was added at a concentration of 0.01 *M* for its catalytic effect, and the pH was controlled by using a glycine solution of pH 9.0. The enzymatic activity is expressed according to the proposal of Liubimova and Engelhardt as  $Q_P$  which represents the microliters of a hypothetical gas (H<sub>3</sub>PO<sub>4</sub>) equivalent to the micrograms of P liberated by 1 mg. of myosin in 1 hr. (8). Protein concentration was measured in the Beckman spectrophotometer by the method of Warburg and Christian (9) or in later experiments by the Kjeldhal procedure.

#### *Superprecipitation Reaction*

A portion of a fresh batch of once-crystallized myosin prepared at pH 6.5 by the method of Szent-Györgyi (10) was dialyzed at 0° in distilled water until chloride-free. The volume of the myosin solution remained nearly unaltered during dialysis. Small aliquots of the dialyzed and the nondialyzed myosin were then precipitated at 25° with the amount of 0.1% acetic acid which it had been determined was required to bring the pH to  $5.3 \pm .05$  as measured with the glass electrode. After appropriate intervals, 0.01 *M* sodium barbital was added to bring the pH to  $6.85 \pm .1$ , and the myosin was tested for superprecipitation with actin and ATP. The results of a typical experiment (Table I) show that undialyzed myosin loses its characteristic reactivity with actin and ATP after a few minutes of isoelectric precipitation whereas dialyzed myosin retains activity though allowed to stand precipitated for several hours. Dialyzed myosin also withstood acidification better in regard to the persistence of the slight precipitation occurring before addition of ATP.

TABLE I

*Ability of Dialyzed and Nondialyzed Myosin to Withstand Isoelectric Precipitation*

0.34 ml. and 0.52 ml. of 0.1% acetic acid were added to 2.0 ml. of dialyzed and non-dialyzed myosin, respectively. Solutions were brought to pH 7 with 0.45 ml. of 0.01 *M* and 0.33 ml. of 0.05 *M* sodium barbital, respectively, and to 0.6 *M* with respect to KCl. The test was made with 0.2 ml. of myosin solution + 0.4 ml. of 0.6 *M* pH 6.9 sodium veronal buffer + 0.1 ml. polymerized actin solution + 2.0 ml. distilled water + 0.1 ml. of 0.3% sodium ATP.<sup>a</sup>

Dialyzed myosin		Nondialyzed myosin	
Time Precipitated at pH 5.3	Actomyosin super- precipitation <sup>b</sup>	Time Precipitated at pH 5.3	Actomyosin super- precipitation <sup>b</sup>
<i>h</i>		<i>h</i>	
0	++++	0	++++
2/3	++++	1/6	+++
3/4	+++	1/3	++
1-1/6	+++	1/2	+ to ++
2-1/2	+++	11/12	0
3-1/6	++ to +++		
3-1/2	++ to +++		
4-3/4	++ to +++		
5-2/3	++		
5-1/2	++		

<sup>a</sup> These proportions were found from testing to give optimal superprecipitation for the solutions used.

<sup>b</sup> Density, contractility, and rapidity of formation of precipitate were judged arbitrarily by gross inspection.

*Enzymatic Activity of Isoelectrically Precipitated Myosin*

A portion of the chloride-free myosin diluted 40-fold in distilled water and gently stirred was left isoelectrically precipitated 18 hr. at 0°. This myosin lost little or none of its reactivity with actin and ATP.

The possibility existed that myosin—still active physiologically after several hours at pH 5.3 in the cold—might be relatively inactive enzymatically. On several occasions dialyzed myosin was left precipitated at pH 5.3 in the cold (0–5°) with 20 to 40-fold dilution in distilled water for a period of 2–15 hr. This treatment was found to have no marked effect on the phosphatase activity, however. The results of two experiments using myosin prepared according to the method of Bailey (11) or Szent-Györgyi are shown in Table II. In each

test one aliquot of myosin was diluted with 20 volumes of cold water. It was acidified and allowed to stand 2 hr. before separation in the refrigerated centrifuge. Another portion was treated similarly after making the salt concentration 0.4 *M* with KCl. A third portion was centrifuged immediately after acidification, resuspended in 20 volumes of water, and dissolved by addition of 0.1% acetic acid to a pH of 4.2. After 10 min., the protein was reprecipitated by adding 0.1 *M* sodium barbital to pH 5.3 and was allowed to stand 30 min. before centrifugation. These samples brought to pH 7 with sodium barbital and dissolved in 0.6 *M* KCl were then tested for ATP-ase activity along with a portion of the unprecipitated myosin. As is seen in Table II the activity of the various preparations was comparable.

TABLE II

*Adenosine Triphosphatase Activity of Myosin Acidified in the Presence or Absence of Salt at 0-5°*

The phosphatase test was carried out with  $0.4 \pm 0.03$  mg. protein and about 3  $\mu$ moles sodium ATP in 2.0 ml. of solution. Enzymatic activity is shown for two dialyzed myosin preparations before and after isoelectric precipitation as described in the text.

Myosin treatment	Q <sub>P</sub> Experiment	
	1 <sup>a</sup>	2
Unprecipitated	1307	1600
Precipitated at pH 5.2	1388	1700
Precipitated at pH 5.2 Dissolved at pH 4.2 Reprecipitated at pH 5.2	1214	2400
Precipitated at pH 5.2 after addition of KCl to 0.4 <i>M</i>		1900

<sup>a</sup> The myosin was prepared by Bailey's Method (11).

An experiment was carried out to determine whether the decline of enzymatic activity under conditions which favor deterioration of the superprecipitation reaction occurs more readily in the presence or absence of salt. Here a myosin twice-crystallized by dilution at pH 6.5 (10) was dialyzed salt-free, and equal portions were brought to pH 5.0 for 20 min. at 26°, one of them in the absence of salt and the other after addition of KCl to 0.4 *M*. It was found on two occasions that the rate of phosphate splitting and the superprecipitation reaction both

deteriorated to a lesser extent in the dialyzed myosin. The decline in the phosphatase test coincided with the decline of the superprecipitation test. This finding is in harmony with the observation of Bailey and Perry that oxidation of the —SH groups of myosin results in a corresponding decrease in ability of the protein to split ATP and to give normal viscosity values with ATP (12). Our findings are in apparent disagreement with those of Buchthal *et al.* (13) who noted no impairment of contractility in actomyosin threads of low enzymatic activity. However, it is possible that the contractility of such threads remains until the myosin, as judged by phosphate-splitting, is nearly completely inactivated. Furthermore, it may be that once the combination of actin and myosin is established as in these threads, contraction may occur although the myosin is altered to such an extent that it would no longer combine with actin and ATP in solution. A similar interpretation may be offered for the discrepancy between our results and those of Singher and Meister (2). However, it may be noted in this connection that in our experience myosin prepared by a short extraction (10) has equally good enzymatic activity whether extracted at pH 5.5, 6.5, or 7.5. In the study referred to, the possibility also exists that the actin which may be presumed to have been present could have contributed to the double refraction of flow independently of changes in the myosin.

A final series of experiments was undertaken to evaluate the effect of actin on the deterioration of myosin when held at the isoelectric point at 26° or when dried from the frozen state. Two portions of myosin, one after addition of 2 volumes of F-actin (5) and the other after a comparable amount of 0.1 *M* KCl were brought to pH 5.2 with 0.01% acetic acid keeping dilution equal in both samples. At intervals an aliquot of each sample was neutralized with 0.1 *M* sodium barbital, brought to a final fivefold dilution, made 0.4 *M* with respect to K<sup>+</sup>, and put on ice until tested for superprecipitation and ATP-ase activity. As shown in Table III, actin greatly protects myosin against the effect of isoelectric precipitation at room temperature. However, as was found previously with myosin, deterioration in the physiologic integrity of actomyosin occurs simultaneously with decline in the enzymatic activity. It is noteworthy that when the rate of splitting of ATP reaches a low level, the superprecipitation, while not unusual otherwise, forms rather slowly, indicating perhaps a dependence of the precipitation on the lysing of ATP.

TABLE III

*Effect of Isoelectric Precipitation on Myosin and Actomyosin*

Phosphatase tests were made with 0.07–0.29 mg. of myosin and about 3  $\mu$ moles ATP in 2.0 ml. of solution. Superprecipitation of actomyosin was tested by diluting 0.5 ml. of actomyosin solution with 1.5 ml. of water and 0.1 ml. of 0.3% sodium ATP. Myosin was tested for superprecipitation by adding 3 volumes of water, actin at 0.5 the amount by weight, and ATP.

	Time at pH 5.2	Q <sub>P</sub>	Density of superpre- cipitate	Comments on precipitate
Myosin	0	800 <sup>a</sup>	++++	Appears within 15 sec.; contracts quickly to a plug.
	1/6	410	++++	Appears within 15 sec.; contracts quickly to a plug.
	1/3	210	+++	Appears within 15 sec.; contracts quickly to a plug.
	3/4	150	+	Slowly developing; noncontractile.
	3	50	0	
Acto- myosin	0	850 <sup>a</sup>	++++	Appears in 10–15 sec.; shrinks quickly to a plug.
	1/4	650	++++	Appears in 10–15 sec.; shrinks quickly to a plug.
	1/3	400	+++	Appears in 10–15 sec.; shrinks quickly to a plug.
	3/4	380	+++	Appears in 10–15 sec.; shrinks quickly to a plug.
	1-1/6	280	+++	Appears in 10–15 sec.; shrinks quickly to a plug.
	1-3/4	250	+++	Slower to develop.
	3-1/2	250	+++	Develops slowly and is less contractile.
	5-1/2	200	+	Noncontractile.

<sup>a</sup> Value is low presumably because in this experiment a commercial ATP preparation containing inhibitors was used.

A preparation of myosin B—*i.e.*, extracted actomyosin (11)—tested in like manner gave results quite in line with those obtained with the synthetic actomyosin.

A synthetic actomyosin preparation was frozen in a Dry Ice–acetone mixture and dried by lyophilization. Aliquots, thawed and dissolved in 0.6 *M* KCl at intervals over a period of 5 days, showed not more than a 25% loss in ability to hydrolyze ATP and no impairment in super-

precipitation. A myosin solution in 0.6 *M* KCl, dried similarly, was found to have lost little or no enzymatic or physiologic activity when redissolved several hours later.

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#### SUMMARY

As regards loss of ability to react with actin and to hydrolyze ATP, myosin withstands isoelectric precipitation at room temperature better in the absence of than in the presence of KCl. The enzymatic and physiologic activities decline comparably in either case. Myosin remains active enzymatically and physiologically after prolonged isoelectric precipitation in the cold.

Myosin precipitated isoelectrically at room temperature is markedly protected against loss of the superprecipitation reaction and loss of ATP-ase activity by actin. Superprecipitation, although slower to occur as the enzymatic activity of isoelectrically precipitated actomyosin is lost, does not fail to appear until over 80% of the enzymatic activity is gone. Actomyosin and myosin withstand drying from the frozen state without marked deterioration of either enzymatic or physiologic properties.

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# The Separation of Pentose and Desoxypentose Nucleic Acids from Isolated Mouse Liver Cell Nuclei <sup>1</sup>

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## INTRODUCTION

A number of workers have now isolated nuclei from various tissues making use of the procedures of Stoneburg (1), Marshak (2), or Dounce (3). The lipides have been separated from such isolated nuclei and rather exhaustively analyzed (1, 4). In addition, such nuclei have been analyzed for their content of total nucleic acid (5) and of both desoxypentose nucleic acid (DNA) and pentose nucleic acid (PNA) (6, 7). It is the purpose of this communication to describe the composition of nuclei, isolated from mouse liver by a modification of the technique of Marshak, and to detail a method whereby the PNA and DNA of such isolated nuclei may be obtained in purified form.

Liver cell nuclei isolated by these procedures have a great preponderance of DNA over PNA, and consequently if separation is attempted by the rather drastic method of Schmidt and Thannhauser (8) there is some contamination of PNA by DNA. The principal innovation that has been introduced has been to obtain by a mild extraction procedure a sample in which PNA accounts for most of the phosphorus and then on this partially purified preparation to employ a somewhat more drastic procedure for final separation. This has enabled us to obtain nuclear PNA in a state of purity sufficient for reliable use in tracer studies involving radiophosphorus.

<sup>1</sup> Aided by grants from the American Cancer Society and the University of Minnesota Graduate School.

## MATERIALS AND METHODS

Normal male mice of the ZBC line<sup>2</sup> between 1 and 2 months of age were used. They were fasted 18–24 hr.<sup>3</sup> following which the livers were removed under ether anesthesia after clamping the portal vein to allow for drainage of blood. The livers were harvested into an iced beaker, forced through a cold tissue press with holes about 1 mm. in diameter, and homogenized 2–3 min. in 4 volumes of ice-cold saline<sup>4</sup> (0.85% NaCl containing 2 ml. 0.1 *N* NaOH/l.). Homogenization was carried out in a Potter-Elvehjem tube (9) and an aliquot of the resulting homogenate removed for estimation of total PNA and DNA by the method of Schneider (10).

The remaining homogenate is spun 4 min. at  $1400 \times g$  in a Sorvall angle centrifuge operated in the refrigerator. The resulting supernatant fluid or "cytoplasmic extract" (11) may be used for separation of cytoplasmic constituents if desired. The sediment is resuspended in cold 2% citric acid and a series of sedimentations and resuspensions in the cold citric acid are carried out. These sedimentations are effected in a clinical centrifuge developing a field of about  $500 \times g$ . The first spin is for 10 min., then 2 spins of 5 min. each, about 5 spins of 3 min. each, and 2 or three 1-min. spins. At about this stage a scum, rich in nuclei, rises to the surface and it becomes necessary to spin 1 min., then stir the surface and spin 1 min. more, to avoid significant loss of nuclei. The process of washing in citric acid is continued until the sediment appears homogeneous and the supernate has only a faint turbidity. This point is reached after about 16 washes in the cold 2% citric acid.

The validity of this end point is strengthened by two observations. Microscopic examination of a smear in dark field reveals only an occasional particle that is not identifiable as a nucleus and even these may be nuclear fragments. The nuclei are intact and clean of any recognizable cytoplasmic tabs. Secondly, it has been observed in experiments in which inorganic radiophosphorus is injected intraperitoneally an hour prior to sacrifice that the specific activity of the subsequently isolated nuclear PNA is many times greater than that of the cytoplasmic PNA. Thus, any cytoplasmic contamination of the isolated nuclei would give a lower specific activity to the "nuclear" PNA. Early experiments, in which nuclei were washed only 8–10 times in citric acid, gave a "nuclear" PNA with an appreciably lower specific activity than that obtained from nuclei washed 15–16 times in citric acid. Further washing in citric acid, up to 25 times, has failed to give a further increase in the specific activity of the nuclear PNA.

After the citric acid washes, the nuclei are washed twice with saline to remove citric acid and then are extracted with 95% alcohol, followed by 3 extractions with boiling alcohol-ether (3:1), and a final alcohol extraction. The pooled alcohol and ether extracts are concentrated almost to dryness and re-extracted with petroleum ether for determination of phospholipide phosphorus.

<sup>2</sup> We are indebted to Dr. J. J. Bittner for supplying the mice used in these experiments.

<sup>3</sup> Fasting facilitates the separation of cytoplasmic constituents, but does not affect the isolation of nuclei.

<sup>4</sup> If cytoplasmic fractionation is not desired then homogenization can be carried out directly in cold 2% citric acid.

The lipide-free nuclei are resuspended in an ice-cold buffer at pH 10 prepared by mixing equal volumes of 0.1 *M* Na<sub>2</sub>CO<sub>3</sub> and 0.1 *M* NaHCO<sub>3</sub>. After standing 30 min. in an ice-water bath<sup>5</sup> the suspension is spun in the clinical centrifuge 2-3 min. and the gelatinous sediment washed twice with the cold buffer.<sup>6</sup> The pooled supernate and washes from the pH 10 extract are precipitated by making up to 5% with cold trichloroacetic acid (TCA). The precipitate is spun down and washed with 5% TCA, then dissolved in a small volume of 0.1 *N* NaOH and heated to 80°C. for 10 min. in order to render the PNA acid-soluble.<sup>7</sup> After heating, the solution is cooled and made 0.1 *N* to HCl and 5% to TCA to precipitate the protein and the small amount of DNA contamination. This acid-soluble fraction represents the nuclear PNA.

In order to obtain the DNA in relatively pure form, the gelatinous sediment remaining after pH 10 extraction of the lipide-free nuclei is resuspended in a small volume of 10% NaCl and placed in a boiling water-bath for 20 min. to render acid-soluble the PNA still associated with this fraction. Then more 10% NaCl is added and heating continued for another 30 min. to coagulate the protein and extract the DNA. The suspension is spun and the sediment re-extracted twice with hot 10% NaCl. The pooled 10% NaCl extracts are treated with 2 volumes of absolute alcohol. After 2 hr. at room temperature the insoluble DNA is spun down and washed with 80% alcohol, then reheated 30 min. with 10% NaCl to remove traces of protein, and the extract again precipitated with alcohol and washed. The precipitate is now dissolved in a small amount of 0.1 *N* NaOH and heated 20 min. at 80°C. to remove last traces of PNA, cooled and made 0.1 *N* to HCl and 5% to TCA. The precipitate is washed with TCA and then hydrolyzed with 5% TCA (10) by heating 30 min. at 80°C. This final supernatant fluid is the DNA.

The bulk of the nuclear protein is obtained from the residue left after extraction with 10% NaCl. This residue is first hydrolyzed with 5% TCA to remove traces of DNA and then dissolved in alkali and aliquots removed for nitrogen analyses.

### *Analytical Procedures*

Total phosphorus was determined according to the method of Fiske and SubbaRow (12) and nitrogen according to the Pregl micro-Kjeldahl method (13). Pentose was estimated by the orcinol-HCl reaction essentially as described by Brown (14), and desoxypentose by the diphenylamine reaction of Dische (15). Absorption of ultraviolet light was determined with the Beckman spectrophotometer.

### RESULTS

During the isolation of nuclei the principal loss occurs during the first few centrifugations in citric acid. It is probable that the yield

<sup>5</sup> It is essential that the temperature be kept low and the time rather short or an appreciable amount of the PNA will be rendered acid-soluble.

<sup>6</sup> After the rather prolonged isolation in citric acid the nuclei retain their morphology subsequent to lipide extraction and suspension in the cold pH 10 buffer. Therefore, the bulk of the DNA sediments in a low gravitational field.

<sup>7</sup> Heating for 2 min. in 0.1 *N* NaOH at 80°C. renders 75% of the PNA soluble in HCl-TCA. Heating for 5 min. renders it all soluble.

TABLE I  
Composition of Isolated Nuclei

	DNA	PNA	Phospho- lipide <sup>b</sup>	Protein <sup>c</sup>	DNA-P PNA-P	PNA-P Lipide-P
	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg.</i>		
1 g. wet liver	2.85 (4) <sup>a</sup> 2.5-3.1	9.11 (4) 7.9-11.2	30.1 (3) 29.5-30.3	126.3 (3) 123-31	0.33	0.12
Nuclei present in 1 g. liver <sup>d</sup>	2.85	0.36 (4) 0.28-0.43	0.35 (4) 0.24-0.52	6.9 (4) 6.4-7.4	3.3	2.5

<sup>a</sup> Figures are averages of the number of experiments shown in parentheses. Ranges are shown below each average. Each experiment represents pooled livers of 12-20 mice.

<sup>b</sup> Phospholipides calculated as lipid P  $\times$  25.

<sup>c</sup> Protein calculated as protein N  $\times$  6.25.

<sup>d</sup> These values are calculated from recovery of DNA in isolated nuclei. Isolated nuclei contained 66.5-77.7% of total cell DNA. If PNA or protein are extracted from nuclei during isolation then the native nuclei in 1 g. of liver would contain more of these constituents than shown.

could be somewhat improved by increasing the speed or time of these centrifugations. Nevertheless, the over-all recovery is 73% based on the DNA in the isolated nuclei compared to that in the original homogenate. The composition of these isolated nuclei is indicated in Table I.

### Purified DNA

The purification of DNA from the isolated nuclei involves very little loss so that 95% can be recovered in purified form. These purified samples of DNA had a nitrogen to phosphorus ratio of 1.74 (range 1.72-1.77), and an  $E_{1\text{cm}}^{1\%}$  at 266  $m\mu$ , based on the assumption of 10.05% phosphorus, of 321. The density of the color developed with the diphenylamine reagent<sup>8</sup> per microgram of phosphorus was 0.022 at 600  $m\mu$ , while the densities of the color developed with the orcinol-HCl reagent<sup>9</sup> per microgram of phosphorus were 0.011 at 660  $m\mu$  and 0.0088

<sup>8</sup> Four ml. of reagent, made by dissolving 1 g. of diphenylamine in 100 ml. of glacial acetic acid and 2.75 ml. of conc.  $\text{H}_2\text{SO}_4$ , is added to 2 ml. of the solution to be tested, heated in a boiling water-bath 10 min., cooled, and read in the Evelyn colorimeter with a 600  $m\mu$  filter.

<sup>9</sup> Four ml. of reagent, made by dissolving 0.2 g. orcinol in 56 ml. conc. HCl and 0.2 ml. 10%  $\text{FeCl}_3$ , is added to 2 ml. of the solution to be tested, heated 15 min. in a boiling water-bath, cooled, and read in the Evelyn colorimeter with the filters at 660 and 565  $m\mu$ .

at 565  $m\mu$  (see Ref. 10). The comparable values for the color developed with PNA per microgram of phosphorus in the orcinol reaction were 0.13 at 660  $m\mu$  and 0.04 at 565  $m\mu$ . Thus, contamination of the DNA fraction with PNA would raise the ratio of the densities of the colors developed by the orcinol and diphenylamine reactions, and would also raise the ratio of densities at 660  $m\mu$  and 565  $m\mu$  in the orcinol reaction. Further purification of the DNA samples failed to change these ratios, and it was therefore assumed that so far as these chemical analyses could suffice the DNA samples were free of PNA. Other work to be reported later, in which radiophosphorus was used, indicates that DNA samples prepared in this way may still contain traces of other phosphorus compounds.

### *Purified PNA*

The extraction of PNA from the lipide-free nuclei by means of the pH 10 buffer has given only about a 50% yield<sup>10</sup> of which about 11% fails to precipitate with the TCA. The material that precipitates with

TABLE II  
*Micrograms of P in Purified Nuclear PNA from 1 g. of Wet Liver*

Exp. no.	Total P	Calculated from pentose ( $\times 2$ ) <sup>a</sup>	Calculated from C V. absorption <sup>b</sup>
1	9.97	9.85	10.09
2	14.45	13.88	13.98
3	12.35	12.92	12.65
4	8.72	9.03	9.01
Av.	11.4	11.4	11.4

<sup>a</sup> A factor of 2 is used in pentose determinations of PNA because analyses of several purified preparations of yeast nucleic acid and of liver pentose nucleic acids indicate that only 50% of the pentose is measured by the orcinol-HCl reaction.

<sup>b</sup> Calculated from an  $E_{1\text{cm}}^{1\%}$  of 312 at 260  $m\mu$  as determined for purified yeast nucleic acid.

TCA contains about 34% PNA, 6% DNA, and 60% protein. Thus, this procedure has increased the ratio of PNA to DNA 47-fold and the ratio of PNA to protein 10-fold from what they are in isolated intact nuclei. It is on this preparation that the mild alkaline hydrolysis of

<sup>10</sup> Recent experiments show promise of materially increasing this yield by spinning the suspension of nuclei in pH 10 buffer at  $23,000 \times g$  for a few minutes in order to compress the gelatinous sediment.

PNA and subsequent TCA precipitation of DNA and protein are carried out. Probably because of the presence of protein during the mild alkaline hydrolysis, the nitrogen to phosphorus ratio of the purified PNA (acid-soluble fraction) is about 2.2. Attempts to demonstrate DNA in the purified PNA fraction by means of the diphenylamine reaction have been equivocal. If any DNA is present it can be there only in traces. Table II gives some analyses on the purified PNA fraction.

### DISCUSSION

In attempting to separate the nuclear PNA and DNA in pure form, two principles have been followed. First, the washing of the nuclei has been carried to a stage where it seems probable that no cytoplasmic contamination remains. Second, the ratio of the desired substance to other phosphorus-containing substances has been made as great as possible before the last step in purification, involving either alkaline or acid hydrolysis, is carried out.

During the prolonged isolation of nuclei in 2% citric acid, it is probable that all acid-soluble constituents are removed. Whether this involves the removal of any part of the lipide, nucleic acid, or protein of the nuclei is impossible to say from these experiments. It is felt, however, that what remains is purely of nuclear origin. Williams *et al.* (4) found that rat liver nuclei isolated by the method of Dounce (3) contained about 12.5% phospholipide as compared to about 3.4% in our mouse liver nuclei. This may reflect a species difference; however, in earlier experiments where nuclei were washed only 8–10 times in citric acid, we observed phospholipide values about 5 times those found in nuclei washed 15 or 16 times. Also, we observed more PNA in the nuclei washed only 8–10 times, and this PNA had a significantly lower specific activity when radiophosphorus was used. Since the most logical contamination of nuclei would seem to be aggregations of cytoplasmic particulates which contain PNA and a high content of phospholipide (11) and in which the PNA phosphorus has a very low specific activity (16), it seemed probable that the decrease in phospholipide and PNA brought about by the longer washing procedure could be ascribed to a more complete removal of cytoplasmic contamination. It is realized that this leaves unanswered the question as to how much nuclear material may have been leached out by the washing procedure employed.

In our nuclei the DNA amounts to 27.3% of the sum of DNA, PNA, phospholipide, and protein. It would be a slightly lower per cent if a

value for total lipide were available. This may be compared with other reported values for liver nuclei from various species which range from 21.7–30.7% (5, 6, 17, 18).

These results for the DNA content of isolated liver nuclei are in sharp contrast to those reported by Pollister and Leuchtenberger (19) for mouse liver nuclei in fixed sections. These workers estimated the protein to nucleic acid ratio by cytological absorption measurements and concluded that this ratio was about 7 times that observed for isolated liver nuclei. They interpret this to mean that large amounts of protein are extracted from nuclei during the isolation procedures. It certainly remains a possibility that some protein is extracted during isolation. It may be calculated from Table I of this paper that their results would imply that intact mouse liver nuclei contain 38% of the total cell protein. If the results of Pollister and Leuchtenberger are verified it would indicate a surprising concentration of protein in the nucleus since Marshak (2) estimates the mouse liver cell nucleus to occupy only 6% of the cell volume.

With respect to the separation of DNA and PNA from purified nuclei, we have employed the fundamentals of the methods of Schmidt and Thannhauser (8) and Schneider (10). However, we have been forced to modify these methods in such a way that prior to the final step of acid or alkaline hydrolysis, the phosphorus of the desired nucleic acid represents the major part of all the phosphorus present. Thus, instead of employing alkaline hydrolysis on lipide-free nuclei where the ratio of DNA-P to PNA-P is 8.3, we have done it after pH 10 extraction at a point where this ratio is less than 0.2. The possibility of significant contamination is thus markedly reduced. Similarly, before the DNA is obtained by hydrolysis with 5% TCA, it is freed as completely as possible, not only of PNA, but also of protein. This is a particularly critical point, since 1 hr. after injection of radiophosphorus the PNA-P and the protein P have specific activities several hundred times that of the DNA-P. Thus, contamination of the DNA-P by as little as 0.5% with phosphorus from either of the other fractions could more than double the observed activity of the DNA-P.

#### SUMMARY

Nuclei have been isolated from mouse liver tissue by a procedure of differential centrifugation in citric acid somewhat comparable to that

used by Marshak (2). The method gives a 73% yield of nuclei, and the isolated nuclei contain 66% protein, 27% DNA, 3.4% PNA, and 3.4% phospholipide.

Purified DNA can be isolated from these nuclei with a yield of 95% and an N:P ratio of 1.74. Observations with radiophosphorus indicate that this fraction may still be contaminated with traces of phosphorus derived from either PNA or protein.

Purified PNA can be isolated from these nuclei with a yield of about 45%. Analyses of this fraction for total phosphorus, pentose, and ultraviolet absorption indicate that within rather narrow limits (see Table II) all of the phosphorus can be accounted for as PNA. The fraction is contaminated with some non-nucleic acid nitrogen, and shows an N:P ratio of about 2.2.

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# Studies on Protein Synthesis *in Vitro*. III: Further Observations on the Incorporation of Methionine into Liver Protein

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## INTRODUCTION

In previous communications (1, 2) it has been shown that methionine labeled with radioactive sulfur is taken up by the proteins of liver slices. The uptake rate was greatly reduced when the tissue was homogenized or after denaturing the proteins. In addition, tissue slices also converted methionine to cystine which was also incorporated into the protein. Part of the cystine taken up was presumably bound by peptide bonds, but it is also probable from experiments with labeled cystine (3) that some of the cystine was bound to the protein by disulfide or by other types of bonds because a large part of the cystine was removed by treatment with a reducing agent. It therefore became desirable to measure the methionine uptake alone.

The present communication deals with the development of methods to separate methionine sulfur from cystine sulfur and with further investigation of the properties of the system. Particular attention has been given to the problem of showing that the non-cystine sulfur incorporated is that of methionine. The effect of poisons, of anaerobic conditions, and of the level of methionine on the rate of uptake has also been examined.

## EXPERIMENTAL

### *Methods*

Liver slices were prepared and incubated in Krebs medium as described in a previous communication (3). Unless otherwise stated one-half gram of wet tissue was

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<sup>1</sup> With the technical assistance of Julia Rutman.

used in each flask and all experiments were run in triplicate. This amount of tissue is the minimum amount which can safely be employed with the analytical methods used. Smaller amounts give too little benzidine sulfate to be titrated satisfactorily. When substances were added in relatively large amounts to the medium, the concentration of sodium chloride was reduced correspondingly to maintain a constant osmotic pressure.

The methionine used was synthesized by methods previously described (4). The experiments are controlled by "zero time blanks," which test the efficiency of the washing procedure and show whether the methionine is of the requisite purity. In this test, slices are placed in the medium containing the labeled methionine, acetate buffer is added, and the proteins immediately denatured by rapidly heating the mixture to the boiling point. The rest of the procedure is carried out as described in later paragraphs. With good samples of the amino acid the blank is very low, but some preparations give very high blanks. The high values are attributable to the presence of homocystine or homocysteine in the methionine sample and are reduced by repurification to remove the contamination. Such contamination need only exist at a very low level in order to make the methionine entirely unsuitable for tissue-slice experiments.

To determine the extent of incorporation of methionine, slices are usually incubated for 2 hr., then boiling 1 *M* acetate buffer at pH 4.5 is added to the flasks and the contents are boiled. The denatured protein is homogenized in the medium in a Potter all-glass apparatus (5) and is separated by centrifugation. Homogenization and washing are repeated 4 times using acetate buffer, pH 4.5. The residual protein is hydrolyzed with 25 ml. of 8 *N* hydrochloric acid for 15 hr., then the acid is removed in the steam-bath. Cystine is precipitated from the hydrolyzate by taking the residue up in 40 ml. of water containing 0.15 ml. of glacial acetic acid, adjusting to pH 3, heating to a boil, and adding cuprous oxide<sup>2</sup> (6). The solution is allowed to cool over a period of 30–40 min., following which the mercaptide is centrifuged and the supernatant fluid (assumed to contain methionine as the only sulfur compound) is poured into a 100-ml. Kjeldahl flask. The methionine sulfur is converted to sulfate by the previously described perchloric acid digestion (7). The digestion should be prolonged for at least 12 hr. During the first 2 hr. most of the nitric acid is boiled off slowly. When the digestion is completed any residual perchloric acid is removed over a Meker burner until even the neck of the flask is thoroughly dry. If this is not done, residual oxidizing agent will cause the formation of objectionable colored products from the benzidine. The black residue in the flask is dissolved in a few milliliters of 12 *N* hydrochloric acid and re-evaporated to dryness. Sulfate is precipitated as the benzidine salt from the residue dissolved in 10–15 ml. of water, using an excess of the benzidine reagent (8) and sufficient acetone to make the final solution 25% by volume. Precipitation is allowed to proceed overnight whereupon the precipitate is collected and washed on Whatman No. 1 paper supported on a demountable Büchner type of filter apparatus (3) so that the precipitate is centered in the middle of the paper on an area of approximately 4.6 cm.<sup>2</sup>

<sup>2</sup> The cuprous oxide used may either be prepared fresh from Fehling's solution made preferably with cupric chloride rather than the sulfate, or else the *brick red* bottled variety may be used. Other dark-colored preparations are less satisfactory.

The radiocativity and equivalence with respect to standard base (0.01 *N* NaOH) are determined directly on this precipitate. Thus the specific activity of the sulfur, defined as the counts/min./ $\mu$ mole of sulfur, is obtained. The tubes used for counting are of the large diameter end-window type without supporting grids and filled nearly to atmospheric pressure.<sup>3</sup> Windows vary in thickness between 1.8 and 2.6 mg./cm<sup>2</sup>. Counting is done for a sufficient length of time to reduce the statistical error to approximately 3%. Where necessary, corrections for self-absorption are made using a standard curve as reference. The absorption data agree with those found by Henriques and co-workers (9). The absorption increases about 1% in going from 0.30 to 0.46 mg. of benzidine sulfate per cm.<sup>2</sup>—the range in which the weights of most of the precipitates lie. Consequently, little correction is usually required.

## RESULTS

A test was made to show the validity of the assumption that the non-cystine sulfur in the digest is all methionine sulfur. One gram of tissue slices was labeled with methionine in the usual manner, the proteins were washed and subjected to hydrolysis. Cystine was precipitated, and to the supernatant fluid was added 500 mg. of methionine. Aliquots were removed, and the specific activity of the methionine sulfur was found to be 101. To the remaining solution was added 5-mg. amounts of cystine, homocystine, thiolhistidine, cystathionine, lanthionine, and djenkolic acid. The methionine was then separated and recrystallized twice from hot water and four times from an alcohol-water mixture. This recrystallized material showed a SA of 89.5, that is, there was no decrease in the value except that which could have resulted from retention of traces of the carriers used. Thus no significant amounts of the substances added could have been present in radioactive form in the supernatant fluid from the hydrolysis of the proteins. A second experiment gave similar results.

When large numbers of samples are included in any one experiment, it is necessary to cut slices from the livers of several animals. If all the slices are pooled, then more than an hour may elapse before sufficient accumulate (10–15 g.) in the pool to suffice for the whole experiment. Under these conditions the activity of the slices suffers a considerable diminution in spite of the fact that the pool is kept in an ice-bath. An alternative method is to take slices from each rat liver and put up several sets of flasks as the animals are sacrificed, and using staggered controls. Analysis of a large body of data obtained using both of these

<sup>3</sup> Our best thanks are due to the staff of Crocker Laboratory, University of California, for making these tubes available to us.

methods is shown in Table I. From these results it is seen that more reproducible results are obtained using the "individual rat" method. Needless to say this procedure does not at all obviate difficulties which may arise due to differences in the rats used such as those due to their genetic constitutions (2).

A specific effect of keeping slices in an ice-bath is seen in the data given in Table II. Slices kept at 0–5° either in the pool or in the flasks for any length of time suffer a great diminution of activity. In 2 hr. the uptake amounts to only 29% of that originally observed when the slices are transferred from the pool to the incubation flasks containing fresh medium. When no transfer is involved the activity is not so

TABLE I  
*Comparison of Tissue Slice Methods*

	Expts	Samples/expt	Coeff of variation <sup>a</sup>
Pooled slice method	13	3	13.8±2.8
Individual rat method	12	3	7.8±1.6

<sup>a</sup> Errors are standard deviations.

greatly reduced and is 56% of the control. Therefore the slices may lose some component or components of the synthetic system to the pool during the 0–5° treatment because activity is partly restored to the system by adding the pool medium (supernatant) to the incubation flasks (Table II, last line). At 0° the uptake is not detectable. This is shown by the following data obtained by incubating slices in a medium containing a somewhat higher concentration of methionine.<sup>4</sup> All the flasks were first incubated for 1 hr. at 37°, after which time the proteins showed a SA of 7.5. This was followed by incubation for an additional hour at temperatures of 0–5°, 25°, or 37°, which resulted in final SA's of 6.9, 8.7, and 12.6, respectively. Thus no additional methionine was taken up in the extra hour at 0–5°, although there was incorporation at 25° and, of course, at 37°. Incidentally in this experiment the observed SA after 3 hr. was 23.9.

It should be noted that there is an apparent inconsistency in the results. The uptake observed in 4 hr. is twice that in the 2-hr. period

<sup>4</sup> The concentration was 0.66  $\mu$ moles/ml. and the SA was also different from that used in the experiments reported in Table II, namely,  $1.5 \times 10^4$  counts/min./ $\mu$ mole. Hence the results are not directly comparable.

TABLE II

*The Effect of Slice Age on Uptake*Methionine used: 0.268  $\mu$ moles/ml. medium with SA:  $3.75 \times 10^4$  counts/min./ $\mu$ mole

Conditions	SA average Counts/min./ $\mu$ mole
2 hr. at 37°	89.3
4 hr. at 37°	186
2 hr. at 0° without methionine, plus 2 hr. at 37°	50.3
1 hr. in pool at 0-5°, plus 2 hr. at 37° in fresh medium	77.8
2 hr. in pool at 0-5°, plus 2 hr. at 37° in fresh medium	25.9
2 hr. in pool at 0-5°, plus 2 hr. at 37° with 1 ml. of supernate from pool in fresh medium	37.7

(Table II), and in the experiment mentioned in the preceding paragraph the 3-hr. uptake is three times the 1-hr. uptake, so that there is no evident falling off in the activity of slices at 37°. These results are also confirmed by an experiment in which slices were incubated for various lengths of time the results of which are shown in Fig. 1. There is apparently a linear increase in the amount of methionine taken up with time during the 4-hr. period of the experiment. Thus there is a falling off in the activity of slices at 0° but none at 37°. It should also

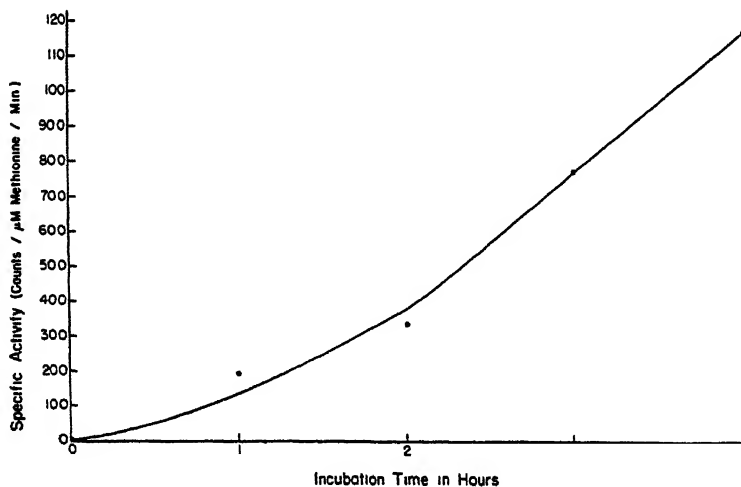


FIG. 1. The uptake of methionine into the proteins of liver slices as a function of the time of incubation. Methionine: 0.268  $\mu$ moles/ml.; SA,  $3.73 \times 10^4$  counts/min./ $\mu$ mole.

be observed in connection with these data that even during the longest period of incubation there is no significant bacterial contamination in the flasks. By plating on agar, only about a hundred organisms/ml. were found after 4 hr. of incubation.

The effect of several respiratory poisons, of anaerobic conditions, and of homogenizing the tissue is shown in Table III. It is quite obvious that the poisons as well as the homogenization all decrease the uptake very greatly. Experiment 5 shows that the washing procedure is satisfactory since this zero time sample only shows 1% of the uninhibited uptake. Obviously, the zero time control is not an entirely satisfactory measure of the methionine which may be adsorbed on the

TABLE III

*The Effect of Poisons, Anaerobiosis, and of Homogenizing Liver Tissue on the Uptake of Methionine by the Protein in vitro*

Time - 2 hr. Medium = Krebs with 0.2% glucose. Labeled addendum = Methionine - S<sup>35</sup>, 0.268  $\mu$ moles/ml. medium with SA  $3.73 \times 10^4$  counts/min./ $\mu$ mole.

Expt. no.	Conditions and concentrations of addenda	SA <sup>a</sup> average	Inhibition
	<i>M</i>	<i>counts/min./<math>\mu</math>mole</i>	<i>per cent</i>
1	Controls	103 $\pm$ 3.5	
	0.001 NaCN	5.5 $\pm$ 0.7	95
2	Controls	63 $\pm$ 10.2	
	0.001 NaN <sub>3</sub>	36 $\pm$ 3.7	43
	0.01 NaN <sub>3</sub>	1.6 $\pm$ 0.5	97
3	Controls	76 $\pm$ 4.0	
	95% N <sub>2</sub> , 5% CO <sub>2</sub>	4.2 $\pm$ 0.9	94
4 <sup>b</sup>	Control slices	76 $\pm$ 5.3	
	Homogenate	6.7 $\pm$ 0.2	91
5 <sup>c</sup>	Control	56 $\pm$ 3	
	0 time incubation	0.6 $\pm$ 0.6	(99)

<sup>a</sup> The errors given are standard error of the mean.

<sup>b</sup> The values given are for the total homogenate of the tissue. Fractionation of the material probably gives particulate preparations which are more active per unit of original tissue.

<sup>c</sup> Second set of samples simply mixed with methionine then proteins immediately denatured—test of washing procedure, *etc.*

protein since there may be a time factor in the adsorption process. An attempt was made to test this point further by an independent method. A large batch of slices was labeled in the usual manner, and the protein washed as described. The homogenized residue was dissolved in a solvent of the following composition: Urea, 5 *M*; potassium chloride, 1 *M*; and sodium carbonate, 0.025 *M*. The preparation was then subjected to dialysis against the same solvent which was changed frequently. This process was continued for 5 days at 10°, after which the protein was reprecipitated by readjustment to pH 4.5 and dilution. Two additional washings were then made before determining the sulfur and the radioactivity of the protein. The results are shown in Table IV. It is seen that the procedure led to some loss of methionine sulfur (24%) but that there was an increase in its specific activity (19%). Consequently, the sulfur lost must have had, if anything, a lower specific activity than that remaining, hence it could not have been loosely bound methionine of high specific activity such as that originally used to label the slices.

TABLE IV

*The Effect of Dialyzing Protein Derived from Labeled Liver Slices  
on the Total Sulfur and on the Labeled Sulfur*

Incubation conditions: As in Table III. Dialysis conditions: medium, alkaline urea at 10°; time, 5 days.

	Methionine sulfur (in protein) $\mu$ mole	SA average counts/mn./ $\mu$ mole
Controls	11.8 $\pm$ 0.47	75.5 $\pm$ 4.0
Urea treated	9.0 $\pm$ 0.19	89.5 $\pm$ 3.3

The effect of changing the concentration of the methionine in the medium is shown in Fig. 2. Experiments of two kinds were done to obtain the results shown. The points on the lower curve were obtained using methionine of high specific activity diluted with inactive DL-isomer, whereas those in the upper curve were obtained when the high SA material was diluted with L-methionine. The curves obtained in this way appear to be characterized by two phases. First there is a phase during which small changes in methionine concentration result in marked differences in uptake, which is followed by a change to a phase where a change in concentration has a much smaller effect. The phenomenon has proved to be reproducible in the hands of several workers using different samples of methionine. The average data from

5 experiments give the following numerical results expressed in terms of per cent replacement, *i.e.*, units of sulfur replaced per unit of sulfur as methionine in the protein. At the levels of 1.33, 4, 16, and 28  $\mu$ moles of methionine/ml. the values are 0.49, 0.59, 0.97, and 1.08, respectively, for 2-hr. periods of incubation. That is at the highest concentration of methionine used (28  $\mu$ moles/ml.) about 0.5% of the tissue methionine is replaced per hour. The animals used were either on a stock diet and starved 18–24 hr. prior to use, or else were on a diet containing no

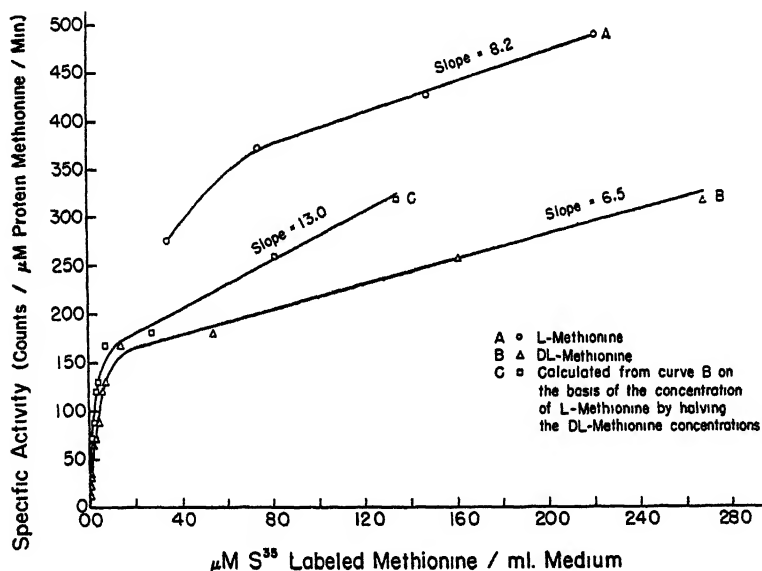


FIG. 2. The effect of the concentration of methionine on its uptake by the proteins of liver slices. Curve A, labeled-methionine diluted with L-isomer. SA L-isomer,  $1.56 \times 10^4$ , SA D-isomer  $2.98 \times 10^4$  counts/min./ $\mu$ mole. Curve C, labeled-methionine diluted with DL-isomer. SA L-isomer  $1.87 \times 10^4$  counts/min./ $\mu$ mole.

protein for 3–7 days before sacrifice. Little difference was observed in the uptake in these animals so variously treated. It is to be noted from the average results that the uptake curve tends to flatten off in the region of the highest methionine concentration used.

When the amount of tissue per flask is varied, the results in Table V are obtained. These data show that within the limits of the experiment the amount of tissue makes no difference whatsoever in the specific

TABLE V

*Effect of Amount of Tissue on Uptake*

Time and medium as in Table III. Labeled addendum = methionine —  $S^{35}$ , 1.34  $\mu$ mole/ml. SA:  $1.68 \times 10^4$  counts/min./ $\mu$ mole.

Weight of tissue mg./g. ml.	SA average counts/min./ $\mu$ mole
300	18.7
400	18.7
500	17.6
600	19.1
700	23.1

activity of the methionine sulfur. In other words, the amount of methionine present is not a limiting factor in the experiment. The uptake of the label is proportional, moreover, to the amount of slices.

TABLE VI

*Effect of Various Amino Acids on Uptake*

Incubation conditions—as in Table III. Labeled addendum = methionine —  $S^{35}$ , 0.268  $\mu$ mole/ml. SA:  $1.2 \times 10^4$  counts/minute/ $\mu$ mole.

Substance	Concentration $\mu$ moles/ml.	SA average counts/min./ $\mu$ mole
None	—	35.8
Glycine	2	38.6
Glycine	10	36.4
Aspartic acid	2	37.7
Aspartic acid	10	33.6
Glutamic acid	2	38.0
Glutamic acid	10	25.1
Succinate	20	8.2

The effect of adding various amino acids to the slices was also tested and the results are shown in Table VI. Little effect was noted in most cases, although at a concentration of 10  $\mu$ moles/ml. glutamic acid appears to be inhibitory. At twice this concentration, succinate exerts a strong inhibition.

## DISCUSSION

The decrease in uptake observed when slices are maintained for a pre-incubation period at 0–5° is rather surprising because other authors have observed that under these conditions the respiration remains intact (10).

The results obtained with the respiratory poisons and anaerobic

conditions confirm those obtained by other workers (11, 12, 13) who have shown that in order for the incorporation reaction to proceed the process of respiration must remain intact. Hence, the incorporation of amino acid does not involve processes which are simply the reverse of hydrolysis. A supply of energy must be available to drive the synthetic reaction. The case of lysine uptake by the particulate fraction from guinea pig liver is exceptional in this respect (13). That an energy supply is required is also shown by the effect of dinitrophenol which likewise inhibits the uptake of labeled amino acid (12).

The implication to be drawn from these results is that the process being measured in these experiments involves the incorporation of amino acid molecules presumably by the formation of new peptide bonds and does not involve the adsorption of the amino acid by the protein. This is also shown by the retention of the label in spite of extensive washing and dialysis procedures. Moreover, it has been shown by other workers (12) that when carboxyl-labeled alanine is used the activity associated with the protein cannot be removed by the ninhydrin reaction. The observations that the rate of incorporation falls off as the slices age, and when the tissue is homogenized, also point in the same direction. Aging would not be expected to affect adsorption phenomena.

The effect of changing the concentration of methionine in the medium on the rate of uptake may serve to indicate that two different processes of methionine incorporation are involved. This is substantiated by the fact that no linear relationship exists between the logarithm of the concentration and the uptake. However, it should be mentioned that part of the rapid rise at the beginning of the curve is no doubt due to the increase in SA of the methionine in the slices. That is, there is a certain amount of preformed methionine in the slices which, at low levels of added labeled methionine, will cause an appreciable dilution of the label. Nevertheless, this cannot be the whole explanation because an entirely similar phenomenon is observed with a well washed particulate fraction from the liver (15).

The differences in the results obtained, depending on whether the methionine used, is diluted with the DL-isomer (curve *B*) or the L-isomer (curve *A*) is to be attributed to the inhibition of the uptake of the L-isomer by its antipode. (Curve *C* is plotted from the data used in curve *B* except that the concentration of the L-isomer only is used, the D- being disregarded.) If this assumption is correct, then it appears that

the inhibition of the uptake of the D-isomer is very considerable. Some uncertainty is introduced into the conclusion because of the possible conversion of the D-isomer into the L- during the course of the experiment. This would result in an increase in the SA of the L-isomer and cause an apparently high uptake. Such effects are probably not great.

#### ACKNOWLEDGMENTS

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#### SUMMARY

1. Evidence is presented which indicates that the uptake of methionine S<sup>35</sup> by tissue slices is due to the formation of bonds and not due to adsorption.

2. The synthetic system in the slices is partly inactivated in two hours at 0-5°.

3. The process is inhibited by respiratory poisons, anaerobiosis, and homogenizing the tissue.

4. The rate of uptake depends on the concentration of methionine and is independent of the number of slices used.

5. Two different processes may be concerned in the uptake.

6. The D-isomer inhibits the uptake of the L-isomer.

7. The uptake proceeds linearly with time over a period of four hours.

8. In the concentrations tested, glycine, aspartic acid, and glutamic acid have little effect on the uptake of methionine.

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# The Hydrolysis of Vitamin A Ester <sup>1</sup>

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## INTRODUCTION

Vitamin A normally circulating in the blood is largely found in the alcohol form (1). Gray, Morgareidge, and Cawley (2) have shown that, after the oral administration of vitamin A ester, absorption was preceded by hydrolysis in the small intestine and the free vitamin A was then transferred across the gut wall. Re-esterification then took place (3) and as Glover, Goodwin, and Morton (4) have stated: ". . . it is this esterified vitamin A in transport to the liver which, superimposed on the normal vitamin A alcohol content of plasma, gives rise to the typical tolerance curve." These authors have found that in rats given 100,000 I. U. of vitamin A, about 4 days are required for the ester form to disappear from the blood. This fraction, it has been postulated (5), is absorbed by the liver partly as the ester and partly as the free alcohol. The latter, they propose, may be formed by the hydrolysis of the esterified vitamin A by a serum esterase. The object of the present investigation has been therefore, to examine the activity of blood or its components in hydrolyzing the esters of vitamin A.

## METHODS

In general the method used for this investigation was to incubate either freshly drawn oxalated whole blood, serum, plasma, or cells from the human, rat, and rabbit for 3 hr. at 37°C., in the dark, with varying concentrations of free and esterified vitamin A. These values were determined according to the chromatographic method for their separation (6), and compared with those from a similar unincubated sample. The amount or concentration of the ester present before and after incubation was used to determine the degree of hydrolysis that had taken place. A 3-hr. incubation time was arbitrarily selected.

<sup>1</sup> This work was supported in part by grants from Merck and Co., Inc., Milbank Memorial Fund, and The National Vitamin Foundation, Inc.

The hydrolysis of vitamin A ester was first examined in whole oxalated blood. The ester was incorporated into whole blood by the oral ingestion of vitamin A, by adding the water-soluble ester to whole blood, or by the ingestion and direct addition of the water-soluble ester variety.<sup>2</sup> Since normally there is only a small amount of ester present in blood it was assumed that the amount of ester added to each sample was approximately the same as the total ester content determined in the fortified sample prior to incubation.  $\alpha$ -Tocopherol in a concentration of 1:10,000 was added to preserve the vitamin A during the 3-hr. incubation period. The amount of the ester and

TABLE I  
*The Hydrolysis of Esterified Vitamin A by Whole Blood and  
Serum of Different Species*

I. U. of vitamin A in 4 ml. of plasma or serum

Subject	Before incubation			After 3-hr. incubation at 37°C.			Hydroly- sis I. U./hr.	Recov- ery
	Free	Ester	Total	Free	Ester	Total		
Human whole blood								per cent
1 <sup>a</sup>	4	6	10	6	3	9	1	90
2 <sup>a</sup>	3	9	12	7	5	12	1.3	100
3 <sup>b</sup>	7	39	46	31	15	46	8.0	100
4 <sup>b</sup>	8	18	26	16	11	27	2.3	104
5 <sup>c</sup>	7	41	48	19	24	43	5.7	90
6 <sup>c</sup>	6	30	36	17	16	33	4.7	92
Rat whole blood <sup>c</sup>	6	32	38	23	12	35	6.7	92
Rabbit whole blood <sup>c</sup>	5	27	32	18	13	31	4.7	97
Human serum <sup>c</sup>	6	47	53	13	39	52	2.0	98
Rat serum <sup>c</sup>	1	45	46	29	17	46	9.3	100
Rabbit serum <sup>c</sup>	7	43	50	18	30	48	4.3	96

<sup>a</sup> Oral ingestion of 100,000 I. U. of vitamin A.

<sup>b</sup> *In vitro* addition of vitamin A ester plus oral ingestion of 50,000 I. U.

<sup>c</sup> *In vitro* addition of vitamin A ester.

alcohol form was determined prior to incubation in the one sample and after incubation in the second. These determinations were on 4-ml. aliquots of plasma from each sample. A similar investigation was carried out incubating 4 ml. of either human, rat, or rabbit serum with varying amounts of the free and ester form. Blood cells were washed twice with the isotonic saline and also incubated with suspensions of the ester in Locke's solution containing 1:10,000 parts of  $\alpha$ -tocopherol and 1 mg.-% of ascorbic acid.  $\alpha$ -Tocopherol alone was not an adequate preservative, so it was necessary to add a combination of  $\alpha$ -tocopherol and ascorbic acid to protect the vitamin A during

<sup>2</sup> This was contributed by the Endo Products, Inc., as an aqueous dispersion of vitamin A from fish liver oil.

incubation. Vitamin A determinations were made before and after incubation on 4 ml. of the supernatant fluid after centrifugation.

In order to make a comparison between the hydrolytic activity of whole blood and its various components the following experiment was carried out: A freshly drawn sample of human blood was divided into four parts. Part I was whole oxalated blood, part II washed blood cells, part III plasma, and part IV serum. Samples of part I were incubated with esterified vitamin A as described above. The amount of vitamin A in each sample was determined on 4-ml. aliquots of its plasma. Equivalent amounts of washed cells, plasma, and serum (*i.e.*, the amount of cells, plasma, and serum contained in 10 ml. of whole blood) were incubated with the ester as previously described.

### RESULTS

It may be noted from Table I that after incubating human blood with known amounts of free and esterified vitamin A there was an increase in the amount of the alcohol and a decrease in the amount of the esterified fraction. Such a change is indicative of hydrolysis of the ester. This reaction occurred if the ester were added either *in vivo* or *in vitro* as a fish liver oil concentrate. The ability to hydrolyze the latter was also exhibited by whole blood from the rat and rabbit. The hydrolysis for human blood ranged from 1-8 I. U./hr., while rat and

TABLE II

*The Hydrolysis of Esterified Vitamin A by Freshly Drawn Oxalated Human Blood and Corresponding Amounts of Cells, Serum and Plasma*

I. U. of vitamin A in 4 ml. of plasma, serum, or cell-free media

Part of blood	Before incubation			After 3 hr. incubation at 37°C.			Hydrolysis I. U./hr.	Recovery
	Free	Ester	Total	Free	Ester	Total		
I (Whole oxalated)	3	13	16	11	7	18	2	<i>per cent</i> 111
II (Cells)	2	10	12	2	10	12	0	100
III (Plasma)	17	41	58	21	37	58	1.3	100
IV (Serum)	15	47	62	18	43	61	1.3	98

rabbit blood showed 6.7 and 4.7 I. U./hr., respectively.<sup>3</sup> In Table I may also be found the results of incubating serum in the presence of free and esterified vitamin A. It is evident that the serum from man, rat, and rabbit are all capable of hydrolyzing the combined form of vitamin A. The plasma fraction can also carry out this hydrolysis (Table II).

A comparison between the hydrolytic activity of whole blood and its various constituents is presented in Table II. It may be noted that approximately the same amount of vitamin A ester was hydrolyzed per hour by whole blood, and corresponding amounts of plasma and serum. No hydrolysis could be demonstrated by the formed elements of the blood.

### DISCUSSION

It appears obvious from the data presented that blood can hydrolyze the ester of vitamin A. This active principle is found in either the serum or plasma fraction of blood. It is indeed quite likely that the hydrolysis may be carried out by one of the serum esterases known to hydrolyze various fatty acid esters.

This hydrolytic action of blood may partially explain why the vitamin A found normally circulating in blood is largely in the alcohol form. It is of course possible that other tissues may also possess this factor. Liver tissue was examined but no such hydrolysis could be demonstrated (7). The role played by this factor on the various physiological and biochemical functions of vitamin A remains to be investigated. It is possible, for example, that this hydrolytic action may in some way be related to the mechanism responsible for regulating the paradoxical relation noted between blood levels and liver content of vitamin A (8). It may also be postulated that if the alcohol vitamin A is the "functional form" (4), then this factor may be of value for the storage of the free form in the liver as well as the possible hydrolysis of the more commonly stored vitamin A ester and its liberation into the blood stream.

### SUMMARY

1. A factor capable of hydrolyzing the ester form of vitamin A has been demonstrated in the blood from man, rats, and rabbits.

<sup>3</sup> This rate of hydrolysis was in terms of the amount of blood necessary to give 4 ml. of plasma.

2. This hydrolytic factor has been shown to be present in the serum and plasma of blood but not in the cellular elements.

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# Amino Acids and Inorganic Sulfur as Sulfur Source for the Growth of Yeasts

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## INTRODUCTION

Yeast cultures, even those belonging to the same species, show differences in bios requirements and, as recent reports from this laboratory (1,2) have shown, yeasts also differ as regards their ability to utilize the nitrogen and carbon of amino acids. As a result of the study described in the present report we may now add to these characteristics the ability to utilize sulfur from one or more chemical combinations, *i.e.*, inorganic sulfur (sulfate), methionine, glutathione, and cysteine.

The media ordinarily used in yeast microbiological tests contain a sufficient amount of sulfur in the form of inorganic salts. A study on sulfur metabolism of bakers' yeast by Sugata and Koch in 1926 (3) revealed that in an artificial medium containing ample "biose vitamin" inorganic sulfate proved the most available form of sulfur; in a sulfate-free medium,  $H_2S$  (at low concentration) was utilized whereas cystine and cysteine stimulated yeast growth only slightly. Fels and Cheldelin (4) have recently reported the utilization of methionine but not cysteine sulfur by a *S. cerevisiae* yeast.

## MATERIALS AND METHODS

The studies on the sulfur metabolism of yeast followed the same general microbiological procedure as that used in the nitrogen (1) and carbon (2) reports.

### *Solutions*

*Sugar and Salts (SAS No. 1) Solution* (without sulfur and with one-fifth the regular amount of dextrose). One liter contains 40 g. of c. p. dextrose (anhydrous), 2.2 g. of  $KH_2PO_4$ , 1.7 g. of KCl, 0.5 g. of  $MgCl_2 \cdot 6H_2O$ , 0.5 g. of  $CaCl_2 \cdot 2H_2O$ , and 0.01 g. of  $FeCl_3$ .

*Potassium Citrate Buffer.* One liter contains 100 g. of potassium citrate ( $K_3C_6H_5O_7 \cdot H_2O$ ) and 20 g. of citric acid ( $H_3C_6H_5O_7 \cdot H_2O$ ).

*Ammonium Chloride Solution.* Thirty mg./ml. of  $NH_4Cl$ .

The solutions are sterilized by heating in flowing steam for 30 min. on three successive days, and may then be stored at room temperature until used.

*Growth Factor Concentrate.* Two hundred ml. contains 2 ml. of 50 mg./ml. inositol solution, 4 ml. of 10 mg./ml. calcium pantothenate solution, 0.2 ml. of 100  $\mu$ g./ml. biotin solution, 2 ml. of 1 mg./ml. thiamine solution, 2 ml. of 1 mg./ml. pyridoxine solution, and 2 ml. of 1 mg./ml. nicotinic acid solution. This mixture should not be sterilized and may be stored in the refrigerator for about a week.

### *Basal Medium*

A basal medium sufficient for 60 tests is prepared by mixing in the following proportions:

SAS No. 1	150 ml.
Potassium citrate buffer	30 ml.
Ammonium chloride solution	30 ml.
Growth factor concentrate	30 ml.

Four ml. of the above basal medium is added to each test tube. The sulfur compounds which are dissolved in distilled water at a concentration of 10  $\mu$ g. of sulfur/ml. are now added and the volume per tube adjusted to 9.5 ml. with distilled water. The tubes are plugged with cotton, sterilized by heating in flowing steam for 15 min., and cooled rapidly in a water-bath.

### *Yeast Inoculum*

The exact preparation of the yeast inoculum (5) has been described previously. The inoculation per tube consists of 0.5 ml. of sterile saline solution containing approximately 0.07 mg. of moist yeast.

### *Yeast Growth*

The yeasts are grown at 30° C. with shaking, and the extent of growth is measured at 16, 20, 24, and 40-hr. intervals of growth by reading percentage absorption of light on a Lumetron 400 colorimeter fitted for use with 18-mm. O.D. test tubes. The white light used in the Lumetron is suitably reduced in intensity by a filter made of gray glass and wire-screen combination.

## EXPERIMENTAL

### *Growth of Torula utilis on Minimal Sulfur and on Sulfate Supplemented Media*

Table I shows that approximately maximum yeast proliferation is attained at 16 hr. with added  $Na_2SO_4$  whereas in the two controls without sulfur supplementation the growth is greatly retarded. The much lower growth in Control 2 was accomplished by carefully washing

all glassware and thoroughly rinsing it with distilled water just before use. Experimental work showed that the growth obtained in Control 2 was not due to the sulfur of the biotin and thiamine in the medium but rather to the sulfur found as impurities in the salts used. As much as 10 $\mu$ g. of sulfur in the form of biotin or thiamine can be added to the control with no appreciable increase in growth.

TABLE I  
*Growth of T. utilis With and Without Added Na<sub>2</sub>SO<sub>4</sub>*

Sulfur source	Per cent absorption		
	After 16 hr.	After 20 hr.	After 24 hr.
No added sulfur (Control 1)	33	37	42
No added sulfur (Control 2)	13	15	16
Na <sub>2</sub> SO <sub>4</sub> (1 mg.)	81	90	93

*Growth Response of T. utilis to Different Levels of Sulfur as Sulfate*

The increases in the growth of *T. utilis* obtained by adding 1, 2, 4, and 6  $\mu$ g. of Na<sub>2</sub>SO<sub>4</sub> sulfur to the basal medium are shown in Table II. This experiment, carried out in triplicate, also shows the good reproducibility of the method. These values can be averaged, and plotted as a straight line curve on semi-logarithmic graph paper. This microbiological technique under appropriate conditions has been successfully applied to the quantitative determination of sulfur and will be reported at another time.

TABLE II  
*Standard Sulfur Growth Curve*  
*T. utilis*

Micrograms sulfur as Na <sub>2</sub> SO <sub>4</sub>	Per cent absorption								
	16 hr.			20 hr.			24 hr.		
Control	13	16	15	17	18	18	18	20	20
1	21	23	24	26	27	27	28	30	29
2	29	30	30	33	34	36	36	37	38
4	42	40	42	49	47	48	51	51	51
6	50	50	48	57	57	55	59	60	58

*Availability of Sulfur in Inorganic and Organic Compounds  
to a Variety of Yeasts*

In Table III the availability of a number of inorganic and organic forms of sulfur for the growth of 6 yeasts is compared. The inorganic forms of sulfur are all used quite readily and almost equally well with all yeasts. The values for  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  are somewhat low due to the deliquescent nature of the compound which was not dried before use.

TABLE III

*The Availability of Inorganic and Organic Sulfur for the Growth of Six Yeasts*

Sulfur source	<i>T. utilis</i>	<i>Myco- derma</i> <sup>a</sup>	<i>T. cremoris</i>	Rasse M <sup>b</sup>	<i>S. carlsbergensis</i> Hansen var. <i>mandshuricus</i> I	<i>S. cerevisiae</i> Hansen str. <i>ana- mensis</i>
10 $\mu\text{g}$ . sulfur as:	Per cent absorption at 24 hr.					
Control	20	10	7	10	12	11
$\text{Na}_2\text{SO}_4$	71	57	68	54	63	55
$\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$	73	55	60	45	54	47
$\text{NaHSO}_3$	72	59	70	58	52	59
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	71	62	69	57	61	58
Glutathione	70	56	68	11	59	48
L-Methionine	73	54	67	39	52	42
D,L-Methionine	72	55	69	34	43	36
L-Cystine	73	61	14	13	16	14
Cysteine HCl	70	55	14	15	16	17

<sup>a</sup> An asporogenous film-forming yeast, not classified as to species.

<sup>b</sup> A bakers' yeast received from the Institut für Gärungsgewerbe, Berlin, Germany, in 1936.

Large differences occur in the utilization of the sulfur of the organic compounds. Four of the yeasts are incapable of growing on cystine or cysteine sulfur and one of these yeasts is also incapable of using glutathione sulfur. All yeasts use both forms of methionine to almost the same extent. The 3 *Saccharomyces* yeasts show a somewhat lower growth on methionine as compared with that on sodium sulfate.

*Classification of Eighty Yeast Cultures*

In view of the fact that the 6 yeasts in Table III showed such definite differences in their sulfur requirements, a survey of a number of strains and species of yeasts was carried out.  $\text{Na}_2\text{SO}_4$  was the only inorganic form of sulfur included since the preliminary work indicated that there would be little differentiation between the inorganic forms of sulfur. Likewise only cystine sulfur was used since cysteine seemed to show the same growth pattern as cystine.

TABLE IV  
*Sulfur Utilization of Eighty Species and Varieties of Yeast*

Group	Representative yeast	Sulfur source										
		Control	Na <sub>2</sub> SO <sub>4</sub>	L-Methionine	DL-Methionine	Glutathione	L-Cystine	Na <sub>2</sub> SO <sub>4</sub>	L-Methionine	DL-Methionine	Glutathione	L-Cystine
		Per cent absorption at 24 hr.						Evaluation of growth <sup>b</sup>				
A	<i>T. utilis</i>	19 20	67 67	67 67	70 70	68 70	68 69	++	++	++	++	++
B	<i>T. cremoris</i>	8 6	68 67	66 67	70 69	69 67	13 14	++	++	++	++	-
C	<i>S. cerevisiae</i> Hansen strain <i>anamensis</i>	10 11	56 54	42 42	36 36	48 49	14 14	++	+	+	++	-
D	<i>Zygosaccharomyces mengelicus</i>	6 5	43 39	30 30	25 27	4 5	33 38	++	+	+	-	++
E	Rasse M	10 10	52 56	39 38	34 33	9 12	13 13	++	+	+	-	-
F	<i>Willia belgica</i> <sup>a</sup>	1 1	1 1	15 16	19 20	36 34	45 43	-	+	+	++	++

<sup>a</sup> After 40 hr. growth.

<sup>b</sup> ++ = Good utilization.

+ = Intermediate utilization.

- = No utilization.

The 80 species and varieties of yeast were divided on the basis of their sulfur utilization into 6 groups, A to F, as shown in Table IV.

The results are given in duplicate and represent per cent absorption of light after a 24-hr. growth period, excepting Group F which had a 40-hr. growth period. Certain cultures in the other groups were too slow growing to obtain an accurate evaluation at 24 hr. These were allowed to grow for 40 hr. and are indicated in the text. Extent of growth is given for a control set of tubes containing no sulfur, and test sets containing 10  $\mu$ g. of sulfur each, supplied by 5 different compounds. For convenience, the per cent light absorption data are also evaluated as good, intermediate, and no utilization.

Yeasts in Group A, of which *T. utilis* is typical, are capable of using all forms of sulfur to the same extent. Other yeasts classified in Group A are:

*Brettanomyces bruxellensis*, *Candida guilliermondii*, *C. guilliermondii* A.T.C. 9058, *Hansenula anomala* var. *robusta* (40 hr.), *H. lambica* (40 hr.), *Mycoderma*, *Saccharomyces aceris-sacchari*, *S. bailii*, *S. behrensianus* (40 hr.), *S. pastorianus*, and *Willia anomala*. A number of other yeasts, slightly atypical but still showing a similar pattern of sulfur utilization are: *Mycoderma lipolytica* (40 hr.), *Pichia belgica*, *P. mandshurica*, *P. kluyveri*, *S. globosus*, *S. ludwigii* (40 hr.), *Torulopsis duttila* (40 hr.), and *T. pulcherrima*.

Yeasts in Group B, of which *T. cremoris* is typical, are capable of using all forms of sulfur well except cystine which is not used. Other representatives of Group B are: *C. pseudotropicalis*, *S. fragilis*, *Zygosaccharomyces lactis*, and *Z. marxianus*.

Group C is similar to B but growth on methionine is incomplete. *S. cerevisiae* Hansen strain *anamensis* is a good example of this group. All the yeasts classified in Group C, with two exceptions, belong to the genus *Saccharomyces* and are named as follows:

*S. annulatus* Negroni, *S. anomalus* Belgicus, *S. bacillaris*, *S. chodati*, *S. logos*, *S. Tokyo* Nakazawa, *S. tubiformis* (40 hr.), *S. carlsbergensis* Chubut, *S. carlsbergensis* var. *mandshuricus* I, *S. carlsbergensis* var. *polymorphus*, *S. cerevisiae* Hansen str. *batatae*, *S. c. H. var. ellipsoideus* str. *valesiacus*, *S. c. H. str. Froberg* (40 hr.), *S. c. H. Guilliermond*, *S. c. H. var. marchalianus*, *S. c. H. var. pulmonalis* (40 hr.), *S. c. H. str. Orsati*, *S. c. H. Rasse II*, *S. c. H. Sake* (Yabe), *S. c. H. Rasse XII*, and 15 strains of bakers', distillers', and wine yeasts not identified by name but very likely belonging to the great number of varieties, strains, etc. of *S. cerevisiae* Hansen yeasts. Also there are *T. colliculosa* and *T. thermantitoneum*.

*Z. menegelicus* shows the typical characteristics of Group D where glutathione is not used but cystine is used. In Group D there are only

two other representatives. These yeasts were isolated as contaminants in a commercial yeast and a commercial syrup.

Group *E* is similar to Group *C* but neither glutathione nor cystine is used. Rasse M, a bakers' yeast obtained from the Institut für Gärungsgewerbe, Berlin, Germany, in 1936, typifies this group. Other cultures of yeasts belonging to Group *E* are: *S. c. H. var. onychophila*, *S. c. H. Nat. Cult. Coll. 6479* (40 hr.), *S. c. H. sp. 152 IFG*, and 5 bakers' and distillers' yeasts not identified as to name but believed to be strains of *S. cerevisiae* Hansen. A few other yeasts showing slightly atypical characteristics are *Kloeckera brevis*, *Pseudosaccharomyces austriacus*, and *T. mucilaginous*.

Group *F* shows *Willia belgica*, a yeast that is incapable of growing on  $\text{Na}_2\text{SO}_4$  but is capable of using both glutathione and cystine. It seemed possible that this yeast might be able to grow on less highly oxidized forms of inorganic sulfur. Accordingly it was tested with  $\text{NaHSO}_3$ ,  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ , and  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ . However there was no growth with any of these compounds. This group has only the one representative in our yeast collection.

## DISCUSSION

Definite differences in the ability of about 80 strains of yeasts to use a number of sulfur-containing compounds as the sole sulfur source in a medium of known composition have been demonstrated. In general inorganic forms of sulfur, and specifically  $\text{Na}_2\text{SO}_4$  sulfur, are the most readily used. Only one yeast was found to be incapable of reducing inorganic sulfates. Methionine sulfur was used by all the yeasts. However a number of yeasts are incapable of growing when cystine or cysteine is the only source of sulfur.

The pathway that has been proposed for the conversion of methionine sulfur to cysteine sulfur in mammals involves the intermediates cystathionine and homocysteine. Studies on the conversion of cysteine sulfur to methionine sulfur by mutants of *Neurospora* (6) and *E. coli* (7) show that the reactions are just the reverse for these organisms. In the present study it was found that yeasts in Groups *A*, *D*, and *F* are able to make either cystine from methionine or methionine from cystine sulfur sources, while those yeasts in Groups *B*, *C*, and *E* can make cystine from methionine sulfur sources but not the reverse. Therefore it would seem that the synthesis by yeasts of cystine sulfur from methionine sulfur is more readily accomplished than the reverse process. It

remains to be determined by which pathway yeasts synthesize cystine from methionine and where the block occurs in those yeasts that cannot use cystine sulfur.

Kirby *et al.* (8) showed that stimulation of aerobic fermentation of bakers' yeast by cysteine or glutathione, which has been reported by others, is mainly due to the hydrogen sulfide arising from the action of yeast on these compounds. Evidently under our more highly aerobic conditions of yeast growth and also with a much lower seeding rate, hydrogen sulfide is not formed or growth would have occurred.

In Table IV we note that Groups *C* and *E* contain all the *Saccharomyces cerevisiae* yeasts which were tested. Differentiation between the many varieties and strains of this important group has always been difficult. It has been particularly difficult to differentiate between those yeasts which require only calcium pantothenate and biotin as growth factors (5) and which have similar amino acid nitrogen (1) and carbon (2) utilization properties. We can now easily divide these yeasts into two groups, those that can and those that cannot grow on glutathione sulfur.

A comparison of the ability of yeasts to utilize the nitrogen, carbon, and sulfur of amino acids shows that the same yeasts exhibit a greater versatility in utilizing the sulfur of cystine and methionine than either the nitrogen or carbon. In the study on nitrogen assimilation (1), only one yeast was found that could utilize cystine at all, while almost one half of the yeasts included in this study could use cystine as a sulfur source. Methionine sulfur also could be used more readily than methionine nitrogen. No yeasts at all were found that could use either methionine or cystine as the sole carbon source (2).

The data in this paper and that in the previous reports on growth factor requirements and on amino acids as nitrogen and carbon sources have been valuable in elucidating a number of problems in yeast metabolism. These experimental results have also been particularly useful in the further characterization of the yeasts in our collection. We believe this information may find general application in yeast taxonomy.

#### SUMMARY

1. Yeasts differ in their ability to utilize the sulfur of organic and inorganic sulfur-containing compounds.
2. A survey of 80 yeasts indicates that the yeasts fall into 6 groups

on the basis of the sulfur compounds they are able to use for growth.

3. The growth of *T. utilis* with definite increments of added sulfur is reproducible and can be plotted as a straight line on semi-logarithmic graph paper, thus paving the way for a microbiological method for sulfur assay.

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# The Maximum Efficiency of Photosynthesis <sup>1</sup>

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## INTRODUCTION

### 1. *Early Experiments*

The efficiency of the energy transformation in photosynthesis

$$E = \frac{\text{chemical energy gained}}{\text{light energy absorbed}}$$

was first determined with the unicellular green alga *Chlorella* in 1922–23 (1). Also introduced into investigations of photosynthesis at that time

<sup>1</sup> For publications on earlier aspects of this investigation, with various collaborators, see Refs. 20, 21, and 22.

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was manometry founded upon the different solubilities of  $O_2$  and  $CO_2$  in aqueous media. The well known equations of the one-vessel method were derived

$$x_{O_2} = h \frac{k_{CO_2} \cdot k_{O_2}}{k_{CO_2} + \gamma k_{O_2}} \quad [1]$$

$$x_{CO_2} = h \frac{k_{CO_2} \cdot k_{O_2}}{k_{CO_2}/\gamma + k_{O_2}} \quad [2]$$

where  $h$  is the observed pressure change and  $\gamma$  is the assimilatory quotient  $x_{CO_2}/x_{O_2}$  = cu. mm.  $CO_2$  exchanged/cmm.  $O_2$  exchanged.  $h$  was measured with an accuracy of a few hundredths of a millimeter by a special cathetometer-microscope focused on a differential manometer that was kept in rapid circular motion.  $\gamma$ , which also had to be known for the computation of the gas exchange  $x_{O_2}$  and  $x_{CO_2}$  by the above equations, was determined by gas analysis; the value of  $-0.91$  was obtained and used in Eqs. 1 and 2.

The problem of the scattering of light by the algal cells, the main source of difficulty in cell-photochemistry, was overcome by the introduction of complete light absorption. Thus, when the intensity of the light entering the manometric vessels had been measured, the absorbed light was obtained by the equation

$$\text{absorbed light} = \text{entering light}.$$

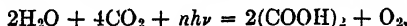
To measure the entering light, a bolometer was constructed with a light-absorbing area greater than the cross section of the light beam used. The total light intensity entering the vessel was then obtained by one single bolometric measurement, and the difficult and inaccurate integration of light intensities over the cross section of the light beam was avoided.

When the foregoing technique had been worked out, a series of 37 efficiency determinations were performed in 3 regions of the visible spectrum, and the manometric and bolometric readings obtained were presented. If the number of quanta absorbed by the chlorophyll of the cells was compared with the  $O_2$  developed, the average quantum number of the series was 4, per molecule of  $O_2$ . Thus, in the red at 660  $m\mu$ , where the molar quantum energy is 43,000 cal., about

$$\frac{112,000}{4 \times 43,000} \times 100 = 65\%$$

of the absorbed light energy was transformed into chemical energy.

It may be emphasized that it is more important to compare the absorbed light with the  $O_2$  developed rather than with the  $CO_2$  consumed. The energy gained per molecule of  $O_2$  is very much the same for most any of the possible end products of photosynthesis, whereas the gain of energy per molecule of  $CO_2$  absorbed could be quite different for various possible end products. As an extreme example, one may note that if one of the end products of photosynthesis were oxalic acid, as in the equation



the gain of energy per molecule of oxygen developed here would obviously be four times the gain of energy per molecule of carbon dioxide consumed.

Efficiencies as high as 65% have thus far not been obtained in any endothermic photochemical reaction. Only one other photochemical reaction is known (2) to have an efficiency approaching that of photosynthesis, the ozonization of molecular oxygen by the wavelength 207  $m\mu$ ,



with an efficiency of  $\frac{68,000}{137,000} \times 100 = 50\%$ . But this exceptionally high efficiency was obtained with quanta of the high energy of 137,000 cal./mole, whereas the high efficiency of photosynthesis was obtained with quanta of the low energy of 43,000 cal./mole, in a reaction that thermodynamically requires at least three such quanta.

Because the photosynthetic efficiency reported in 1923 was very high, doubts arose as to whether the results were correct. Some investigators, in fact, working with different methods (3), succeeded only in realizing much lower efficiencies. The doubts increased when (4, 5) an error was claimed to have been discovered in the method of 1923. It was alleged: that when *Chlorella*, previously kept in the dark, was illuminated for short periods (e.g., 5–10 min.),  $CO_2$  burst out of the cells, and was reabsorbed when the cells were darkened again; that a large part of the pressure changes observed in 1923 in light and in dark were outbursts and inbursts of  $CO_2$  and that only a fraction (one-third to one-half) could have been due to photosynthesis; and that the assimilatory quotient,  $\gamma = CO_2 \text{ exchanged}/O_2 \text{ exchanged}$ , was not approximately minus one ( $-0.91$ ) as determined by gas analysis in 1923 for experimental periods of several hours, but that its real value must have approached  $+4$ . Therefore, the real minimum quantum requirement of photosynthesis must have been not 4 but nearer 12, and the real maximum efficiency not 65% but nearer 22%. This allegation seemed to have been further confirmed by experiments performed not in acid medium as in 1923 but at pH 9 in carbonate buffer; this kept the pressure of  $CO_2$  invariant and therefore manometrically avoided the supposed errors caused by the outburst and inburst of  $CO_2$ . In such nonphysiological buffers, which had been introduced in 1918 (1) for simplification of certain types of experiments, but avoided for determinations of maximum efficiency, quantum requirements of 10–12 per molecule of  $O_2$  were reported (5).

However, the proposed " $CO_2$  outburst," at variance with the experience of the previous century and a half, was never actually demonstrated in published experiments involving quantum yield measurements. In the only completely detailed efficiency experiment in which

the light-dark time course was reported [(4) p. 821], calculations, not performed by the authors, show that the pressure changes for the second 5-minute periods of illumination were actually more positive (less negative) than for the first 5-minute periods ( $-1.86$  for the second period compared with  $-2.17$  for the first;  $+0.11$  for the second period compared with  $-2.13$  for the first). This result, offered as a typical experimental example, was a direct contradiction of an outburst and of [(4) Fig. 2] provided to illustrate an initial pressure burst graphically. In a later paper (5), in which identification of the pressure burst with a burst of  $\text{CO}_2$  was attempted, the only data again available [(5), Figs. 2 and 3] were obtained by the two-vessel method in which the two vessels were studied not simultaneously but 5 hours apart, and the vessel-constant data necessary to calculate cu. mm.  $\text{O}_2$  and  $\text{CO}_2$  from pressure changes were unfortunately not supplied so that it is not possible for the reader to check the calculations independently or to arrive at an estimate of the error involved; the latter ordinarily may be shown to become large as values of  $\gamma$  approach  $-0.5$  to  $0$ , and extremely large for positive values ( $0$  to  $+4$ ).

## 2. Later Experiments

In 1945 (6) the assimilatory quotient  $\gamma = \text{CO}_2/\text{O}_2$  was redetermined for *Chlorella* suspended in acid culture medium, but this time the manometric instead of the gas analysis method was employed, and the illumination periods were of the order of 10 min., not hours. Again a  $\gamma$  value of about minus one ( $-0.93$ ) was obtained.

A new series of efficiency determinations was then carried out with the method of 1923, but this time with different volumes of fluid and gas phases in the vessels. While the relation,  $v_F/v_G = \text{volume of fluid/volume of gas}$ , had been 2.24 in 1923, this time it was 1.24. Again an average quantum requirement of 4 was obtained, when again the experiments were computed with a  $\gamma$  value of  $-0.9$ . According to the principle of the two-vessel method (7), the same quantum requirement could not have been obtained in 1923 and 1945, if the true  $\gamma$  value had differed markedly from minus one.

At the end of 1948, a few two-vessel efficiency experiments were carried out by us at the Botany Laboratory of the University of Illinois, Urbana, through the courtesy of Dr. Robert Emerson, with cultures of *Chlorella* provided by him. The cells, when washed in fresh

acid culture medium and strongly illuminated with an incandescent lamp, produced  $O_2$  like normal cells, *i.e.*, the Blackman reaction was normal. But at low-light intensities of red light, where the photochemical reaction was the limiting reaction, the  $O_2$  production was at first often small or zero, or even negative ( $O_2$  being absorbed in light instead of being produced). Evidently the photochemical reaction was abnormally ineffective, or some photooxidation process was superimposed. After a time, when the cells had been shaken for many hours in the fresh acid culture medium, the photochemical reactivity often recovered. On 3 of 10 days when experiments were carried out, quantum requirements of 4-6/molecule of  $O_2$  produced or  $CO_2$  consumed were obtained after long "latent" periods.

Such experiments left little doubt that quantum requirements as low as 4-6/molecule of  $O_2$  produced or  $CO_2$  consumed were experimentally observable by the two-vessel method under favorable conditions. But the irregular behavior of the cells employed, especially their long latent periods, seemed to us so unsatisfactory that we decided to reinvestigate from the beginning the problem of the efficiency of photosynthesis.

While our new investigation was proceeding in Bethesda in early 1949, a series of papers on the efficiency problem appeared in book form in "Photosynthesis in Plants" (8). The results reported in this volume seemed to be conclusive and final: with three different and independent methods—manometric, polarographic, and calorimetric—minimum quantum requirements of 10-12/molecule of  $O_2$  produced were obtained.

## NEW EXPERIMENTS

### 1. Cultivation of *Chlorella*

A strain of *Chlorella pyrenoidosa*, isolated and identified many years ago by Dr. Florence Meier of the Smithsonian Institution, was cultivated in Drechsel gas washing bottles containing 200 ml. of the following salt solution: 5 g.  $MgSO_4 \cdot 7H_2O$ , 2.5 g.  $KNO_3$ , 2.5 g.  $KH_2PO_4$ , 2 g.  $NaCl$ , and 5 mg.  $FeSO_4 \cdot 7H_2O$  containing Zn impurity, in 1 l. of filtered unsterilized well water (pH of the solution 4.5-5.0). The cultures were maintained at room temperatures of 25-30 C. and were aerated with 5%  $CO_2$  in air at a rate of about 500 cc. gas/min., rapid enough to prevent cell sedimentation, and were constantly illuminated with a 100-watt incandescent lamp at a distance of about 30 cm. The inoculum was 100 cu. mm., sometimes even 200 cu. mm., of cells per flask. When after a few days the cells had multiplied to 600-800 cu. mm., they were washed in fresh culture medium in a No. 2 International Centrifuge at the lowest speed giving nearly complete settling in about 10 min., and were

then either reinoculated or used for efficiency determinations. Bacterial growth was found to be negligible, due to the acid reaction, the lack of added organic matter in the salt solution, and possibly antibiotics produced by the *Chlorella*.

Cultivated by this method, used for many years by one of us (D. B.), the *Chlorella* had a relatively stable respiration and gave high photosynthetic efficiencies. The main drawback of the old culture method (1) was very likely the cell sedimentation, which unfortunately was praised as an advantage in 1922 and was therefore favored by later investigators. In the course of the years the tall Erlenmeyer flasks originally employed became broader, and the aeration became so slow that the greater part of the cells sedimented and suffered in the sediments from lack of oxygen<sup>2</sup>, carbon dioxide, and light.

There still remains the question as to the seeming superiority of well water over distilled water. Thus far, high photochemical efficiencies have been reported only for *Chlorella* cultivated with tap, well, or lake water, the active substances of which, if any, must be widely distributed, since equally efficient yields have been obtained with waters from Berlin, Liebenberg, Bethesda, and Woods Hole. It is probably safer, for the present, and in the absence of specific investigation, to continue with the use of well or natural waters for the cultures.

### 2. Intermittency of Illumination

When *Chlorella* in optically thin suspensions is illuminated with increasing light intensities, the rate of O<sub>2</sub> production at first increases proportionally to the light intensity, then increases more slowly, and finally, at "saturation intensity," becomes constant. Thus, with increasing light intensities the efficiency of photosynthesis decreases, owing, as is generally assumed, to the limiting influence of a dark reaction. It has long been considered as a major difficulty of efficiency determinations, that the maximum efficiency can only be obtained at very low light intensities; this maximum has to be computed from small pressure changes that involve important "corrections for respiration." It will be shown in the following that this difficulty may be overcome by taking advantage of intermittent illumination with high light intensities.

In the steady state at light saturation, our *Chlorella* produces about 30 times its own cell volume of oxygen gas/hr. at 20 C.; and in the region of proportional inten-

<sup>2</sup> In a recent paper (9) on the culture of *Chlorella*, carried out by the methods of 1922, damage from lack of oxygen was cited as being due to nitrite poisoning. This is not true generally, but only when the salt medium contains free nitric acid [(1), p. 385].

sities it produces several times its cell volume of  $O_2$ /hr. As earlier indicated (11, 12), *Chlorella* and most plants may commonly show a rectangularly hyperbolic function of rate of photosynthesis against light intensity, which means virtual proportionality up to at least 20-30% of maximum rate under favorable conditions [cf. (1), pp. 289, 329].

The *Chlorella* suspensions used for our efficiency determinations were not optically thin, but so dense that the red light was virtually completely absorbed in the suspensions. For example, 300 cu. mm. of cells suspended in 7 ml. of culture medium, were placed in shaken rectangular vessels of 8 cm.<sup>2</sup> bottom area. Imagine now a device whereby the 300-cu. mm. cells could be homogeneously illuminated as in optically thin suspensions, then the 300-cu. mm. cells at saturation intensity would produce  $30 \times 300 = 9000$  cu. mm. of  $O_2$  gas/hr., and hence at proportional intensities, at the very least, 1000 cu. mm.  $O_2$  gas/hr. This means that maximum efficiencies could be determined at light intensities that produced as much as 1000 cu. mm. of  $O_2$ /hr.

This is not the case, however, when the cell suspension is at rest during the illumination. Then the cells in the outer layers of the suspension are more strongly illuminated than in the inner layers and the oxygen production is unequally distributed over the 300-cu. mm. cells. The cells in the inner layers might produce no  $O_2$  at all, and some cells in the outer layers would produce much more  $O_2$  than three times their cell volume per hour. Thus in the outer layers the rate of  $O_2$  production may be less than proportional to the light intensity, and the efficiency, determined for the total cell suspension, could not be the maximum efficiency.

But when the cell suspension is shaken and the cells move in cycles from higher to lower intensities and vice versa, then in a time that is large compared with the duration of a 1-cell cycle, all of the 300-cu. mm. cells may absorb the same amount of light, and produce, proportional to the light intensity, the same amount of oxygen. In this state the maximum efficiency can be obtained.

To reach this state the motion of the cells must be so rapid that the light energy absorbed in any fraction of time in any part of the cell suspension does not diminish the concentrations of the limiting dark reactions. The more a given total light intensity is concentrated in space and the stronger the spectral region of the light is absorbed by the cells, the smaller will be the illuminated space inside the cell suspension, and the more rapid therefore must be the shaking to distribute the light evenly over the cells of the suspension.

For example, when a narrow beam of red light was sent into a suspension of 300-cu. mm. cells, maximum efficiency was obtained in one instance up to an  $O_2$  production of 70 cu. mm./hr., but less than maximum efficiency was obtained when the light intensity of the narrow beam was doubled, the motion of the cells being insufficient for the higher light intensity. When on the other hand the greater part of the cell suspension was illuminated with diffuse white light, maximum efficiency was obtained from the initial beam of red light up to an over-all  $O_2$  production of 500 cu. mm./hr. as effected by both light sources. This approached an oxygen production of two times the cell volume per hour, a value up to which proportionality to the light intensity may be readily observed in the steady state in experiments with optically thin suspensions.

These facts suggest that the efficiency of photosynthesis should be determined by illuminating cell suspensions from all sides in diffuse

light of a spectral region that is only weakly absorbed by the cells. But even with the help of the new actinometric technique (13), this procedure would not allow a correct measurement of the absorbed light. Only when a collimated narrow light beam enters the cell suspension, and the spectral region is strongly absorbed by the cells, is a correct measurement possible. Thus, as a compromise, diffuse white light whose intensity was not measured was used to compensate or over-compensate the respiration, and then we measured the efficiency of additional photosynthesis induced by a relatively narrow beam of red light of measured intensity.

This section may be concluded with an equation that clarifies the advantageous nature of the intermittency of the illumination in rapidly shaken, dense cell suspensions. If cells are illuminated *continuously* by light of the intensity  $i_o$ ,  $\left[ \frac{\text{quanta}}{\text{minutes} \times \text{cm.}^2} \right]$ , then the equivalent *intermittent* intensity  $i_z$  is given by the equation

$$i_o = i_z \frac{\Delta t_1}{\Delta t_1 + \Delta t_2}, \quad [3]$$

where  $\Delta t_1$  is the time of illumination and  $\Delta t_2$  is the time of darkness.  $\frac{\Delta t_1}{\Delta t_1 + \Delta t_2}$  may be called the "intermittency factor." It is the fraction of time the cells are illuminated in a rapidly shaken dense cell suspension. It is an oversimplification, of course, to assume that a given cell alternates only between the full incident intensity  $i_o$  and complete darkness, whereas all the intermediate intensities actually occur.

The intermittency factor is the smaller, the smaller the illuminated volume  $v_{\text{light}}$  of the cell suspension and the greater the dark volume  $v_{\text{dark}}$  of the cell suspension. Approximately,

$$\frac{\Delta t_1}{\Delta t_1 + \Delta t_2} = \frac{v_{\text{light}}}{v_{\text{light}} + v_{\text{dark}}}, \quad [4]$$

and when we insert [4] in [3] we obtain

$$i_o = i_z \times \frac{v_{\text{light}}}{v_{\text{light}} + v_{\text{dark}}}, \quad [5]$$

an equation which allows one to estimate  $i_z$ , approximately, from magnitudes that can all be easily determined experimentally.

### 3. Compensating Light Intensity

*Chlorella* in optically thin suspensions may be illuminated with such a light intensity (which depends upon the temperature of the cells and the wavelength of the light) that the  $O_2$  production of photosynthesis equals the  $O_2$  consumption of respiration. This is the "compensating" light intensity for  $O_2$ . The same light intensity compensates the  $CO_2$

production of respiration only if the respiratory and the assimilatory quotients,  $\text{CO}_2/\text{O}_2$ , are equal. As a rule they are not exactly equal and so the compensating light intensities for  $\text{O}_2$  and  $\text{CO}_2$  are not exactly equal. This can easily be demonstrated in experiments with the two-vessel method. When the pressure change in one vessel is brought to zero by a certain light intensity, the pressure change in the other vessel is as a rule not brought to zero, but may be either somewhat positive or negative. Generally "compensating intensity" in the following means compensating intensity with respect to  $\text{O}_2$ .

If the  $\text{O}_2$  consumption of a dense cell suspension is compensated by light, it is possible that in a part of the cells respiration is overcompensated and in another part undercompensated. But if the suspension is so rapidly shaken that the incident light intensity is evenly distributed over all the cells, then when no  $\text{O}_2$  enters or leaves the cell suspension as a whole, no  $\text{O}_2$  enters or leaves any cell in any part of the suspension. This state is therefore a state of true, not of apparent compensation, and is explained by Eq. 3, where  $i_c$  is the compensating light intensity for thin cell suspensions.

#### 4. Mechanism of Compensation

Compensation of respiration by light has been generally explained by the  $\text{O}_2$  production of photosynthesis. But again and again in the history of the science of photosynthesis the question has been discussed as to whether light did not inhibit the very process of respiration itself, for example, by catalytically inactivating respiratory enzymes or by reducing intermediates of respiration (14). We have investigated this problem by the following type of experimental procedure:

In a rectangular vessel, bearing two side-arms, was placed 300 cu. mm. of *Chlorella*, suspended in 6 ml. of acid culture medium of pH 4.9. The side-arms contained NaOH, the gas-space air. The suspension was illuminated by a beam of red light (630–660  $m\mu$ ) which entered the cell suspension in a vertical direction through the bottom of the vessel. The total intensity of the beam was about 0.25 microeinsteins/min., and the cross section of the beam at the bottom of the vessel was 3 cm.<sup>2</sup> The main part of the light was absorbed in the first millimeter depth of the suspension, so that the illuminated volume of the cell suspension was only 0.3 cu. mm. or 0.05 of the total volume of the cell suspension.

When the vessel was shaken rapidly, no compensatory effect of the light was observed, that is, the negative pressure changes in the light and in the dark were virtually equal. When on the other hand the gas space contained 5%  $\text{CO}_2$ , everything else being equal, quantum requirements of 3 to 5 were obtained for aliquots of the

same cell suspension. Such experiments, many times repeated with the same result, proved that light does not inhibit respiration, when employed at intensities capable of giving maximum photosynthetic efficiencies; such light, when it compensates respiration, does so by photosynthesis, the gas exchange of which happens to be the reverse of the gas exchange of respiration.

This result does not contradict earlier observations that in the near absence of  $\text{CO}_2$  in the gas space, light can compensate respiration. The light intensities then employed were much higher and were not controlled by simultaneous efficiency determinations. Moreover, in the present experiments we had the advantage of adequate intermittent illumination. In their cycles from dark to light and vice versa the cells had time to give off in the longer dark periods most of the  $\text{CO}_2$  produced in their respiration, and when they entered the illuminated volume of the cell suspension they were relatively free of  $\text{CO}_2$ . Thus in the competition between light and alkali for the  $\text{CO}_2$  produced in respiration, our special set-up worked in favor of the alkali.

### 5. The Significance of Compensation

Cultivated as described, *Chlorella* has shown under experimental conditions at 20° an initial respiration of 0.5 to 1.2 times its own volume of  $\text{O}_2$  gas/hr. After long manometric experiments values as low as 0.2 times the cell volume of  $\text{O}_2$  gas/hr. have been observed. Thus very different rates of photosynthesis may be necessary to compensate respiration and the "compensating light intensity" is a highly variable magnitude.

Compensation of respiration is important for the following reasons:

1. When photosynthetic efficiencies were determined manometrically below the compensation point by a sequence of dark and light periods, the experiments had to be discontinued after some hours, because not only the respiration, but also the efficiency of photosynthesis usually decreased. But when the respiration was overcompensated, so that the pressure changes throughout the whole duration of the experiment were definitely positive, then the efficiency of photosynthesis remained constant for even more than 24 hr. Compensation of respiration therefore made long experiments possible.

2. Compensation does not eliminate respiration chemically; but it eliminates respiration manometrically, and thus removes many of the difficulties of efficiency determinations. Positive pressure changes can be produced in the desired range. They are more constant than negative pressure changes and when the light actions are obtained as differences

of two positive pressure changes at two intensities, the accuracy of the manometric method is improved.

3. Although it has been shown that light does not inhibit respiration, the theoretical possibility still remained that the process of photosynthesis might inhibit respiration. Thus the old problem, of whether respiration in the dark and during photosynthesis is the same, was still important in experiments below the compensation point. But the problem no longer exists when efficiencies are determined as differences of two positive pressure changes at two light intensities. For then, at the lower intensity photosynthesis has already had occasion to interfere with respiration, leaving no further respiration for the higher intensity to interfere with.

4. Thermodynamically, efficiency determinations below the compensation point were uncertain because below this point chemical energy is not gained, but only the loss of chemical energy is inhibited. But when in experiments above the compensation point we obtain the same quantum requirement as below this point, it is proved that one molecule of  $O_2$  produced or one less molecule of  $O_2$  consumed are thermodynamically equivalent.

5. Let us consider a *Chlorella* suspension to be a machine that transforms light energy into chemical energy. The maintenance of this machine requires an expenditure of energy that is defrayed by respiration and amounts to 112,000 cal./mole of  $O_2$  consumed. If we subtract this amount from our gain of energy, we obtain what may be termed the "economic efficiency,"  $E'$ , as distinguished from the "thermodynamic efficiency,"  $E$ .

When, for example, the quantum requirement is 4, the thermodynamic efficiency in red light is

$$E = \frac{112,000}{4 \times 43,000} \times 100 = 65\%,$$

but the economic efficiency is

$$E' = E \times \frac{f-1}{f},$$

where  $f$  is the "compensation factor,"

$$f = \frac{\text{oxygen developed in light}}{\text{oxygen consumed in the dark}}.$$

At the compensation point we have  $f = 1$ , and therefore  $E' = 0$ .

At 6 times the compensation point ( $f = 6$ ), where we still obtain a thermodynamic efficiency of 65%, the economic efficiency is

$$E' = 65 \frac{f-1}{f} = 65 \times \frac{5}{6} = 54\%.$$

At 10 times the compensation point ( $f = 10$ ), if the thermodynamic efficiency were still 65%, the economic efficiency would be

$$E' = 65 \frac{f-1}{f} = 65 \times \frac{9}{10} = 59\%.$$

Thus by experiments above the compensation point it can be shown that the economic efficiency of photosynthesis is high, even when the energy spent by nature for the maintenance of the energy-transforming machine is subtracted from the thermodynamic gain.

6. The idea has recently been discussed that nature may possibly utilize the energy of respiration by diverting it in the light to photosynthesis, away from functions it has in the dark (14). If so, then the efficiencies computed for the absorbed light energy should be different at different degrees of compensation. When respiration is compensated sixfold, it could contribute to the production of 1 mole of  $O_2$  only  $112,000/6 = 18,000$  cal., that is, about 11% of the absorbed light energy of 4 quanta of red light. But when respiration is but once compensated, it could contribute about 65% of the absorbed light energy. The fact that the same efficiencies of light are observed for uncompensated as for sixfold compensated cells seems to preclude the possibility that the energy of respiration contributes significantly to photosynthesis.

The foregoing discussion shows how firm has become the foundation for the new efficiency determinations based on experiments above the compensation point. Most objections, procedural as well as theoretical, that have been raised concerning high photosynthetic efficiencies since 1923, have in one way or another centered around respiration. By compensating it, these objections have lost not only their quantitative significance but even their qualitative meaning.

### 6. *The Two-Vessel Method*

By the two-vessel method, introduced in 1924 (7), the  $O_2$  exchange as well as the  $CO_2$  exchange of cells can be determined manometrically in the presence of indefinitely high pressures of  $CO_2$ . Equal amounts of cells are placed in two vessels, in each of which  $v_F/v_G$ , the ratio of the volume of the liquid phase to that of the gas phase, is different. Then the same gas exchange produces different pressure changes in the two vessels and from these different pressure changes the quotient  $\gamma = CO_2/O_2$  and the gas exchanges  $x_{O_2}$  and  $x_{CO_2}$  may be calculated.

It is a necessary condition of this method that the gas exchange of the

cells in the two vessels be exactly the same for the whole duration of an experiment. But owing to the principle of the method there must be differences of volume in the two vessels. It is an important question whether these differences of volume can possibly induce secondary differences in the gas exchanges of the cells in the two vessels, during the course of an experiment.

When in two vessels, with equal amounts of cells, 5 and 7 ml. of liquid are placed, the total volume of each of the two vessels being 14 ml.,  $v_F/v_G$  in the one vessel is 0.56 and in the other 1.0. This is an appropriate methodological difference. However, the concentrations of the cells are different. The rate of the intermittency cycles of the cells in the two vessels could be different and so therefore the light actions could be different. In the course of long experiments autoinhibition of the cells might conceivably occur and cause different decreases in the gas exchanges in the two vessels.

Such sources of error are avoided when the volumes of the liquid phases are made equal. Then the total volumes of the vessels must be different to obtain the necessary differences in  $v_F/v_G$ . For example, 18 and 14 ml. total volume and 7 ml. of liquid volume is an appropriate arrangement,  $v_F/v_G$  being in the one vessel 0.64 and in the other 1.0. But then the gas exchange of the cells produces different partial pressures of  $\text{CO}_2$  in the two vessels, with conceivable consequences on the cell metabolism. We prefer this inequality to the inequalities of cell concentrations, because inequalities of  $\text{CO}_2$  pressures, which have yet to be shown to have effects over wide ranges of variation, can easily be readjusted by regassing. Thus, the greater part of our efficiency determinations have been carried out with equal liquid and different gas volumes.

Another source of error is the differential time factor which may occur if the pressure changes produced by light in the two vessels are not observed simultaneously (an exaggerated instance of this was previously discussed in *Early Experiments* with respect to Ref. 5). The ideal would be to illuminate the two vessels simultaneously by two light beams, identical not only in total intensity, but also in geometrical design. We did not have such a pair of beams at our disposal and so we illuminated the two vessels one after another closely together in point of time. We illuminated in periods of usually 10 min., by moving one light beam from one vessel to the other and vice versa and taking the readings for both vessels simultaneously. When this alternation was repeated many times we obtained for every vessel a series of dark values and of light values, or of light values of the intensities  $i_0$  and  $i_0 + \Delta i$ ; and when for each vessel all the dark values were summed up and likewise all the light values, the time factor was virtually eliminated and the pressure changes in the two vessels were obtained essentially simultaneously.

### 7. The Equations of the Two-Vessel Method

These equations, derived in 1924 (7), are adapted here to the special purposes of photosynthesis. Because the experiments with compensated respiration play so important a role in this work, the equations are presented not for a sequence of darkness and illumination, but for a

sequence of two illuminations with two different light intensities, one of which may approach zero as a limit. When the lower light intensity is zero, then the equations hold true for the ordinary sequence of darkness and illumination. The light intensities are denoted as  $J$  and  $J + \Delta J$ . They have the units of quanta/min. and are related to  $i$  by the equation

$$J = i \times \text{cm.}^2 [\text{quanta/min.}].$$

Let both vessels be illuminated first with an intensity  $J$  and then with an intensity  $J + \Delta J$ ; and let the pressure changes be  $h_J$  and  $h_{J+\Delta J}$  for vessel I, and  $h'_J$  and  $h'_{J+\Delta J}$  for vessel II. Then when the pressure changes are observed for equal time periods, and when the two vessels contain the same amount of cells, the gas exchanges  $x_{O_2}$  and  $x_{CO_2}$ , which are equal in the two vessels, can be calculated by the equations:

$$(x_{O_2})_J = \frac{h_J \times k_{CO_2} - h'_J \times k'_{CO_2}}{k_{CO_2}/k_{O_2} - k'_{CO_2}/k'_{O_2}} \quad [6]$$

$$(x_{CO_2})_J = \frac{h_J \times k_{O_2} - h'_J \times k'_{O_2}}{k_{O_2}/k_{CO_2} - k'_{O_2}/k'_{CO_2}} \quad [7]$$

$$(x_{O_2})_{J+\Delta J} = \frac{h_{J+\Delta J} \times k_{CO_2} - h'_{J+\Delta J} \times k'_{CO_2}}{k_{CO_2}/k_{O_2} - k'_{CO_2}/k'_{O_2}} \quad [6a]$$

$$(x_{CO_2})_{J+\Delta J} = \frac{h_{J+\Delta J} \times k_{O_2} - h'_{J+\Delta J} \times k'_{O_2}}{k_{O_2}/k_{CO_2} - k'_{O_2}/k'_{CO_2}}, \quad [7a]$$

where  $k_{O_2}$  and  $k_{CO_2}$  are the simple vessel constants, no prime mark for vessel I and a prime mark for vessel II. All  $h$  values are positive when the light intensity  $J$  overcompensates respiration. All  $h$  values are usually negative when  $J = 0$ , except at high  $\Delta J$  values.

From Eqs. 6, 7, 6a, and 7a we can calculate the action of the light increment  $\Delta J$ , if we assume that the gas exchange measured at the intensity  $J$  continues during the illumination with the intensity  $J + \Delta J$ . Then we may subtract [6] from [6a] and [7] from [7a] and obtain:

$$(x_{O_2})_{J+\Delta J} - (x_{O_2})_J = \frac{(h_{J+\Delta J} - h_J) \times k_{CO_2} - (h'_{J+\Delta J} - h'_J) \times k'_{CO_2}}{k_{CO_2}/k_{O_2} - k'_{CO_2}/k'_{O_2}} \quad [8]$$

$$(x_{CO_2})_{J+\Delta J} - (x_{CO_2})_J = \frac{(h_{J+\Delta J} - h_J) \times k_{O_2} - (h'_{J+\Delta J} - h'_J) \times k'_{O_2}}{k_{O_2}/k_{CO_2} - k'_{O_2}/k'_{CO_2}} \quad [9]$$

and if we write

$$\begin{aligned} y_{O_2} &= (x_{O_2})_{J+\Delta J} - (x_{O_2})_J \\ y_{CO_2} &= (x_{CO_2})_{J+\Delta J} - (x_{CO_2})_J \\ H &= h_{J+\Delta J} - h_J \\ H' &= h'_{J+\Delta J} - h'_J \end{aligned}$$

we obtain instead of [8] and [9]

$$y_{O_2} = \frac{H \times k_{CO_2} - H' \times k'_{CO_2}}{k_{CO_2}/k_{O_2} - k'_{CO_2}/k'_{O_2}} \quad [10]$$

$$y_{CO_2} = \frac{H \times k_{O_2} - H' \times k'_{O_2}}{k_{O_2}/k_{CO_2} - k'_{O_2}/k'_{CO_2}}, \quad [11]$$

where  $y_{O_2}$  and  $y_{CO_2}$  are the gas exchanges effected by the light increment  $\Delta J$ .

There can be little doubt that when the increment  $\Delta J$  is added to a high overcompensating intensity  $J$  the action of  $J$  continues. One might have been more doubtful for the case where  $\Delta J$  is added to dark cells ( $J = 0$ ). But since the same efficiencies have been obtained for  $\Delta J$  whether  $J$  was quite large or zero, the validity of eqs. 10 and 11 is proved.

### 8. The Light and Its Absorption

A Steinheil glass 3-prism spectrograph, operated with a focal length of 195 mm. at  $f/3.5$  for the collimator and a focal length of 710 mm. for the telescope was used as a monochromator. The slit was illuminated with a 750-w. projection lamp. The image of the coiled filament at about  $20^\circ$  to its plane was projected onto the slit with an auxiliary lens. A 1000-w. voltage regulator was used to supply power to the lamp, which operated at constant current.

The width of the entrance slit was about 2 mm., corresponding to about  $20\text{ }\mu$  in the red region. A slit was placed in the focal plane of the telescope and was adjusted to have a width of about  $30\text{ }\mu$  covering the region 630–660  $\mu$ . A lens was placed behind this slit to throw, in a weakly convergent beam, an image of the exit prism face on the bottom of the manometer vessel.

The area of the beam at the vessel was about  $3\text{ cm.}^2$  and the energy flux was about 0.6 microeinsts/min., or in terms of the actinometer 13.4 cu. mm. of  $\text{O}_2$  absorbed/min. This intensity was decreased when desired by placing in the light beam just before the exit slit blackened wire screens calibrated by the National Bureau of Standards.

The beam of red light entered the manometer vessel in a vertical direction from below and was completely absorbed in the cell suspension. Its incident intensity, which therefore was also the absorbed intensity, was measured actinometrically, and is denoted in the following experiments as  $\Delta J$ .

A second light source was a 100-w. incandescent lamp, kept constant by a 500-w. voltage regulator. The lamp was mounted above the thermostat symmetrically to the two vessels and could be moved vertically to produce the desired state of compensation or overcompensation in the cells. The total absorbed intensity of this light is denoted in all experiments as  $J$ . It was not measured,<sup>4</sup> because efficiencies were only calculated for the increment  $\Delta J$  and thus the action of  $j$  was eliminated.

Attention may be called to the different volumes of cells which absorbed the measured red light from below and the unmeasured white light diffusely (mainly from above and the side, also some from below due to reflection back from the tank bottom). When 300 cu. mm. of *Chlorella*, suspended in 7 ml. of liquid, was placed in a rectangular vessel of  $8\text{ cm.}^2$  bottom area, as in most of our efficiency determinations, then the red light  $\Delta J$  was absorbed in a volume of about  $3 \times 0.1 = 0.3\text{ cc.}$ , that is, about 0.04 of the volume of the cell suspension. On the other hand, the white light was absorbed in the greater part of the cell suspension, because the white light entered the cell suspension through a greater surface and was on the average less absorbed by the cells because of the shorter wavelengths also involved.

<sup>4</sup> One must be warned against measuring  $J$  actinometrically.  $J$  may contain a spectral region that is absorbed by the chlorophyll in the cells, but not by ethylchlorophyllide dissolved in pyridine. Other spectral regions are not absorbed completely owing to the incident angles of the white light.

In a discussion (Society of General Physiology, Woods Hole, June 22, 1949) the question was raised as to whether the white light from the lamp above possibly did not materially reach the lowest layers of the cell suspensions, so that the measured red light from below was absorbed in a cell layer of noncompensated respiration, and hence the efficiency calculated for the light increment  $\Delta J$  was possibly still the efficiency of an inhibition of respiration. Although this question, owing to the rapid motion of the cells, had to be answered in the negative, it has been tested experimentally as a result of the discussion. Respiration was also compensated mainly from below, instead of from above, by white light filtered through a red filter that transmitted wavelengths longer than 560  $m\mu$ . This time the increment of measured red light from below was definitely absorbed by the very cells whose respiration was overcompensated. The same efficiencies for the increment  $\Delta J$  were obtained when the compensation was effected by light from below as from above.

In the same discussion objections were raised against the procedure of complete absorption, and experiments with optically thin cell suspensions were suggested. But the methods used to measure light absorption in turbid media seem to be still unsatisfactorily developed in connection with manometry. It may be remembered that 10 years ago Noddack and coworkers (15-19) determined quantum requirements of thin suspensions of *Chlorella* that absorbed only about 10% of the incident light. But Noddack observed quantum requirements of 4 in carbonate buffer, which suggests, according to our knowledge of today, that his light absorption measurements were possibly in error by more than 100%, unless his cultures, which were maintained in an unusual manner, had actually become adapted to give high efficiencies in carbonate buffer.

The only serious objection raised against complete absorption was too high a respiration. But since efficiencies are determined with compensated respiration, this objection is no longer valid.

### 9. The Measurement of the Quantum Intensities

Light measurements were carried out manometrically as described in 1949 (13). Two hundred mg. of thiourea and 3 mg. of ethyl chlorophyllide,<sup>6</sup> dissolved in 7 ml.

<sup>6</sup> Note: The crystallized ethylchlorophyllide was prepared by Walter Christian of the Kaiser Wilhelm Institute at Berlin-Dahlem. Chlorophyll itself was not used owing to the foaming of the preparation at hand in pyridine.

pyridine, were placed in a rectangular vessel of about 8 cm.<sup>2</sup> bottom area, with O<sub>2</sub> in the gas phase. A beam of red light of 630–660 mμ and of a cross section of 3 cm.<sup>2</sup> entered the solution in a vertical direction through the bottom of the vessel and was completely absorbed in the solution. With an adequate rate of shaking, the O<sub>2</sub> consumption of the solution was proportional to the light intensity and did not increase with increasing shaking rates.

Let the decrease in O<sub>2</sub> pressure in the actinometer be  $h$  mm. in  $t$  minutes of illumination and let  $k_{O_2}$  (mm.<sup>3</sup> be the vessel constant for oxygen in pyridine ( $\alpha_{O_2}^{30^\circ} = 0.092$ ), then the total intensity  $\Delta J$  of the light beam is obtained by the equation:

$$\Delta J = h k_{O_2} / 22.4t \text{ (}\mu\text{mole quanta/min.)} \quad [12]$$

where 22.4 is the volume of one  $\mu$ mole of gas in cu. mm. The quantum intensity was usually determined at the order of magnitude of about 1  $\mu$ mole/10 min. (i.e., 22.4 cu. mm./10 min.), as arranged by the use of suitable neutral screens.

Because our application does not require the conversion of cu. mm. of O<sub>2</sub> to  $\mu$ moles of quanta, we may express the light intensities most simply by the cu. mm. of O<sub>2</sub> absorbed/min. in the actinometer

$$\Delta J = h k_{O_2} / t \text{ (cu. mm. O}_2\text{/min.)} \quad [13]$$

### 10. The Measurement of Quantum Requirements

In each of two rectangular vessels of unequal total volume, bearing capillary vents, were placed 300 cu. mm. of *Chlorella*, suspended in 7 ml. of culture medium at pH 4.9–5.1. The vessels were connected to simple Haldane-Barcroft blood-gas manometers (capillary diameter 0.8 mm.) and aerated in the thermostat, usually at 20 C., with 5% CO<sub>2</sub> in air, with a low shaking rate at first. The shaking motion was horizontal with an excursion of 2 cm. After equilibration was reached and the vents closed, the shaking was speeded up to 150/min. and some 15 min. later the first manometric readings were taken. Foaming never occurred, even in experiments of more than 24 hr. duration. Splashing occurred only when by accident a manometer was too loosely fastened and jerked suddenly during horizontal motion.

In all experiments the measured light, the light increment  $\Delta J$ , was of the spectral region 630–660 mμ, isolated by the monochromator. The beam of this red light entered the thermostat in a horizontal direction through a lateral double glass window (Thermopane, to eliminate moisture condensation), and was reflected by a 45 mirror vertically into a manometer vessel. The mirror could be moved horizontally in the thermostat, reflecting the light alternately into the one or the other vessel. In both positions of the mirror the intensities of the light beam at the bottoms of the vessels were sufficiently equal, if the water in the thermostat was clean. Small inequalities were eliminated, if necessary, by interchanging the positions of the manometers.

The subsequent procedure was different depending on whether the experiments were carried out with uncompensated or compensated respiration. In all experiments with uncompensated respiration the mirror was shifted, and thus the gas exchange in both vessels was obtained without interruption, the one vessel being in the dark when the other was in the light. The periods of dark and light varied in these experiments

from 5-60 min. and the duration of the entire experiments did not exceed several hours after which the light action usually began to decrease along with the respiration. Owing to the rapid shaking, physical manometric transition effects of equilibration were not ordinarily observed when dark cells were illuminated, and vice versa, even when the readings were taken every 1 or 2 instead of 5 or 10 min.

In the experiments with compensated respiration the method of shifting the mirror was not usually applied. In a thermostat diffusely illuminated by the compensating white light, shifting of the mirror could alter the distribution of the white light, and the two vessels even if equally illuminated by  $J$  before the shifting might not be after a shifting. Thus only one position in the thermostat could be used for the measurement of the  $\Delta J$  light action, if essentially equal  $J$  values for each vessel were desired.<sup>6</sup> Hence, first one vessel was illuminated alternately with the intensities  $J$  and  $J + \Delta J$ , and in succession the second vessel was brought to the same position and illuminated in the same manner. In the meantime the vessel not receiving  $\Delta J$  was shaken in compensating white light ( $J$ ), but not necessarily read manometrically. These experiments with compensated or overcompensated respiration could be extended for a long time without decrease of efficiency. If after 24 hr. of continuous shaking and illuminating with  $J$  the efficiency began to decrease, transfer of the cells to fresh culture medium raised the efficiency again to the initial value.

When in the efficiency experiments, with or without compensated respiration, the light intensity  $\Delta J$  effected in  $t$  minutes the pressure changes  $H$  in the one vessel and  $H'$  in the other,  $y_{O_2}$  and  $y_{CO_2}$ , the gas exchanges effected by  $\Delta J \times t$  were calculated from  $H$  and  $H'$  by eqs. 9 and 10 and obtained in cu. mm. of gas.  $\Delta J$  was then determined manometrically. The manometer vessels, containing the cell suspension, were replaced by a similar vessel containing, instead of the 7 ml. of cell suspension, 7 ml. of the actinometric solution. Two such vessels may be used, the one to be placed in the red light beam and the other as a control in the "dark." When in  $t$  minutes of illumination  $\Delta J$  effected the pressure change  $h$ ,  $\Delta J$  was calculated by Eq. 12 and obtained in cu. mm. of  $O_2$ /min.

The quotient of the two oxygen exchanges, computed for the same time, is the quantum requirement per molecule of oxygen:

$$\frac{1}{\Phi} = \frac{O_2 \text{ absorbed by the actinometer}}{O_2 \text{ produced by the cells}} = \frac{\Delta J \times t \left[ \frac{h\nu}{O_2} \right]}{y_{O_2}}. \quad [14]$$

$\frac{1}{\Phi}$  is thus obtained by the measurement of three pressure changes. Their

<sup>6</sup> Since experience eventually showed that the action produced by  $\Delta J$  was independent of  $J$  over wide limits, it was not strictly necessary for  $J$  to be equal for both vessels, and in certain types of experiments shifting of the mirror from one vessel to the other was permissible, if distribution of the white light was not significantly altered.

magnitudes were in the range of 25–50 mm. With every new experimental arrangement, it must be checked, both for the cell suspension and the actinometer, that the manometric readings are independent of an increase in shaking rate.

With regard to the outburst and inburst of  $\text{CO}_2$ , inferred during the last 10 years, even if never demonstrated by published data in quantum yield experiments, it may be emphasized that the quantum requirement per molecule of  $\text{O}_2$  obtained by Eq. 14 is independent of all alleged fluctuations of  $\text{CO}_2$ . When from the pressure changes  $H$  and  $H'$  the gas exchange is computed by Eqs. 10 and 11,  $y_{\text{O}_2}$  is obtained independent of the  $\text{CO}_2$  exchange and  $y_{\text{CO}_2}$  is obtained independent of the  $\text{O}_2$  exchange. Measurement of the  $\text{O}_2$  and  $\text{CO}_2$  metabolism by the two-vessel method would be improperly understood if it were insisted, as upon occasion it has been, that the  $\text{O}_2$  uptake after the interruption of illumination could only be measured after 40 min. of dark, by which time the inburst of carbon dioxide would supposedly be completed. On the contrary, for any period of time, during illumination as well as after illumination, the two-vessel method yields the  $\text{O}_2$  exchange independently of all  $\text{CO}_2$  fluctuations, providing, of course, that physical equilibration is adequately maintained.

The described procedure for measurement of photosynthetic efficiency represents a very great simplification of the quantum requirement determination procedures described in 1923 that were adopted in part but never *in toto* by later investigators. The special differential manometer, inconvenient to handle, and the cathetometer-microscope, are replaced by simple Haldane-Barcroft blood-gas manometers and by direct observation. The bolometer, with its manifold auxiliary equipment, is replaced by the simplest of all such energy measuring instruments, the manometric actinometer. Finally, especially when the monochromator is replaced by interference or other appropriate filters, quantum requirements of photosynthesis may be performed as simple classroom experiments, and have been (20).

#### EXPERIMENTAL DETAILS

Our experimental equipment was set up by the middle of March: the Steinheil monochromator; the thermostatted water-bath with Thermopane window, made by the American Instrument Company (Silver Spring, Md.); the manometers and the

rectangular vessels manufactured by E. Machlett and Sons (New York City); and culturing arrangements for growing the strain of *Chlorella pyrenoidosa* that was obtained through the courtesy of Dr. F. E. Allison of the Plant Industry Station (Beltsville, Md.). In the very first experiment we obtained a quantum requirement of 3. But it took us about two and a half months to find out the conditions under which high efficiencies could be obtained in every experiment. In these two and a half months we observed quantum requirements of 3 to 6, but also higher requirements, indeed as high as 20. We often observed rapidly decreasing respirations and light actions in experiments with uncompensated respiration, and could calculate by the equations of the two-vessel method a wide range of uncertain yields because the necessary condition of these equations—identity of metabolism in the two vessels—was not yet invariably fulfilled. Only gradually did we come to clarify and identify the reasons for the irregularities: the sedimentation of the cells in the cultures, the not always adequate shaking of the manometer vessels, the too few cells in the manometer vessels, the too great time differences between manometric readings of the two vessels where  $J = 0$ , the damage done to the cells when shaken too long in the dark, the too high intensity of the measured red-light beam, *etc.* In addition, all our early experiments were carried out at 10°C. (as in 1923) where manometric uncertainties are greater than at 20°C. the temperature finally adopted here.

We therefore decided to report here in detail only experiments performed since May 30th. In these experiments all necessary requirements that we know of were complied with and therefore we felt justified in averaging the results as reported in the *Summary and Conclusions*. Earlier reports of various aspects of the work, with various collaborators, have appeared elsewhere (20, 21, 22). The present work is based upon a background of a total of more than one hundred experiments of the type illustrated in the ten examples shortly to follow. Many minor aspects of the work, and miscellaneous control experiments, have not been detailed, but brief mention may be made of some as follows: (1) The measured red light beam ( $\Delta J$ ) when first filtered through the actinometric solution produced no photosynthetic action. (2) The high photosynthetic efficiency was not decreased in vessels containing Pd catalyst in the side-arm to remove  $H_2$  possibly formed. Mass spectrographic analysis of the vessel gas phase at the end of a prolonged experiment (without Pd in side-arm) indicated no significant occurrence of  $H_2$  or other unusual gases (oxides of nitrogen, *etc.*) (3) The photosynthetic yield in bicarbonate medium with 5%  $CO_2$  in air, at pH *ca.* 7–7.5, was less (*ca.* 8 quanta/ $O_2$ ) than in culture medium at pH 5. (4) In an experiment carried out with the cells washed and taken up in distilled water instead of culture medium, but with 5%  $CO_2$  in air as usual (pH 5), the photosynthetic efficiency remained high, indicating that low pH rather than salt composition is the more important prerequisite for high efficiency. (5) For any period that an illuminated manometer was not shaken, a considerable fraction of the photosynthetic activity might be lost; this was tested for stationary periods of 30 sec. and more. Thus, a vessel alternately shaken and halted every 30 sec. (or every 1, 2, or 5 min) might give during a total elapsed time of 10 min. only about 60–70% of the pressure change shown upon 10 min. continuous shaking; it was not practicable to use periods much shorter than 30 sec., but in this connection rotating sector intermittency experiments are projected.

The letter symbols used in the following description of the experiments signify:

$J$  Intensity of the compensating light absorbed per minute in the cell suspension.  $J$  had to be kept constant during an experiment, but was not measured.

$\Delta J$  Intensity of the added measured red light, incident and absorbed per minute in the cell suspension, measured actinometrically and expressed as cu. mm.  $O_2$ /min.

$h_J$  Millimeters of observed pressure change in  $t$  minutes in vessel I, when  $J \times t$  is absorbed by the cell suspension.

$h_{J+\Delta J}$  Millimeters of observed pressure change in  $t$  minutes in vessel I, when  $(J + \Delta J) \times t$  is absorbed by the cell suspension.

$H$   $h_{J+\Delta J} + h_J$ .

$h'_J$ ,  $h'_{J+\Delta J}$ , and  $H'$  refer, correspondingly, to vessel II.

$\left. \begin{matrix} k_{O_2} \text{ and} \\ k_{CO_2} \end{matrix} \right\} \text{ simple vessel constants (mm.}^2\text{) for vessel I} \left. \vphantom{\begin{matrix} k_{O_2} \text{ and} \\ k_{CO_2} \end{matrix}} \right\} \alpha_{O_2} = 0.03.$   
 $\left. \begin{matrix} k'_{O_2} \text{ and} \\ k'_{CO_2} \end{matrix} \right\} \text{ simple vessel constants (mm.}^2\text{) for vessel II} \left. \vphantom{\begin{matrix} k'_{O_2} \text{ and} \\ k'_{CO_2} \end{matrix}} \right\} \alpha_{CO_2} = 0.87$

$y_{O_2}$  Cubic mm. of  $O_2$  produced by the action of  $\Delta J \times t$  in the cell suspension.

$y_{CO_2}$  Cubic mm. of  $CO_2$  produced by the action of  $\Delta J \times t$  in the cell suspension.

$\frac{1}{\Phi}$  quantum requirement per molecule  $O_2$  produced ( $h\nu/O_2$ ).

$y_{O_2}$  and  $y_{CO_2}$  were computed by use of Eqs. 10 and 11,  $\Delta J$  by use of Eq. 13, and  $\frac{1}{\Phi}$  by use of Eq. 14.

In all experiments the temperature of the thermostat was 20 C. and the spectral region of the added red light of the intensity  $\Delta J$  was 630–60  $m\mu$ .  $\Delta J$  should usually not exceed 6 cu. mm.  $O_2$ /min. (0.27 microeinstains/min.) under our prevailing conditions.

*Experiment 1.*

May 30, 1949

Acid culture medium. 270 cu. mm. of cells in each vessel. 5% CO<sub>2</sub> in air.

$$\Delta J = 5.4$$

Vessel No. 5		Vessel No. 3	
$v = 13.913$	$v_F = 7.00$	$v = 17.993$	$v_F = 7.00$
$k'_{O_2} = 0.665$	$k'_{CO_2} = 1.253$	$k'_{O_2} = 1.046$	$k'_{CO_2} = 1.634$
<i>mm.</i>		<i>mm.</i>	
10' dark - 10.5	10' $\Delta J + 0.5$	10' dark - 2.0	10' $\Delta J + 3.0$
10' dark - 10.0	10' $\Delta J + 0$	10' dark - 3.5	10' $\Delta J + 2.0$
10' dark - 9.0	10' $\Delta J + 1.5$	10' dark - 2.5	10' $\Delta J + 3.5$
10' dark - 8.5	10' $\Delta J + 0$	10' dark - 2.5	10' $\Delta J + 3.5$
10' dark - 9.0	10' $\Delta J + 1$	10' dark - 0	10' $\Delta J + 3.0$
10' dark - 8.0	10' $\Delta J + 1$	10' dark - 1.0	10' $\Delta J + 5.0$
<hr/>		<hr/>	
60' dark - 55.0	60' $\Delta J + 4.0$	60' dark - 11.5	60' $\Delta J + 20.0$
60' $H'$	+ 59 mm.	60' $H$	+ 31.5 mm.

$$\left. \begin{array}{l} y_{O_2} + 70.4 \text{ cu. mm.} \\ y_{CO_2} - 56.0 \text{ cu. mm.} \end{array} \right\} \gamma = -0.8$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 60}{70.4} = 4.6$$

$$\Delta J = 5.4$$

No. 5		No. 3	
Respiration over-compensated		Respiration over-compensated	
5' $J + 5.0$	5' $(J + \Delta J) + 11.5$	5' $J + 6.5$	5' $(J + \Delta J) + 9.0$
5' $J + 6.5$	5' $(J + \Delta J) + 9.5$	5' $J + 6.5$	5' $(J + \Delta J) + 10.0$
5' $J + 6.5$	5' $(J + \Delta J) + 9.5$	5' $J + 7.0$	5' $(J + \Delta J) + 8.5$
5' $J + 5.5$	5' $(J + \Delta J) + 13.0$		
5' $J + 7.0$	5' $(J + \Delta J) + 15.0$		
<hr/>		<hr/>	
25' $J + 30.5$	25' $(J + \Delta J) + 58.5$	15' $J + 20.0$	15' $(J + \Delta J) + 27.5$
25' $H'$	+ 28.0 mm.	25' $H$	+ 12.5 mm.

$$\left. \begin{array}{l} y_{O_2} + 46.3 \text{ cu. mm.} \\ y_{CO_2} - 47.6 \text{ cu. mm.} \end{array} \right\} \gamma = -1.03$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 25}{46.3} = 2.9$$

$$\Delta J = 5.4$$

No. 5		No. 3	
Respiration more over-compensated		Respiration more over-compensated	
5' $J'$ + 18.5	5' ( $J' + \Delta J$ ) + 22	5' $J'$ + 14	5' ( $J' + \Delta J$ ) + 15.0
5' $J'$ + 18.0	5' ( $J' + \Delta J$ ) + 22.5	5' $J'$ + 14	5' ( $J' + \Delta J$ ) + 16.5
5' $J'$ + 16.5	5' ( $J' + \Delta J$ ) + 22.0	5' $J'$ + 12.5	5' ( $J' + \Delta J$ ) + 16.5
5' $J'$ + 17.5	5' ( $J' + \Delta J$ ) + 20.5	5' $J'$ + 14.0	5' ( $J' + \Delta J$ ) + 14.0
5' $J'$ + 17.0	5' ( $J' + \Delta J$ ) + 23.0	5' $J'$ + 11.5	5' ( $J' + \Delta J$ ) + 15.0
		5' $J'$ + 12.0	5' ( $J' + \Delta J$ ) + 14.5
25' $J'$ + 87.5	25' ( $J' + \Delta J$ ) + 110	30' $J'$ + 78	30' ( $J' + \Delta J$ ) + 91.5
30' $H'$ + 27 mm.		30' $H$ + 13.5 mm.	

$$\left. \begin{array}{l} y_{O_2} = + 36.3 \text{ cu. mm.} \\ y_{CO_2} = - 32.6 \text{ cu. mm.} \end{array} \right\} \gamma = - 0.9$$

$$\frac{1}{\Phi} - \frac{\Delta J \times 30}{36.3} = 4.5$$

Total duration of the experiment more than 7 hr. The entire experiment was performed with the same cell suspension. The result is that the quantum requirement is the same below and well above the compensation point, within the experimental error.

### Experiment 2.

June 1, 1949

Comparison of the quantum requirements in acid culture medium, pH 4.9, and 0.1 *M* carbonate buffer (15 vol. 0.1 *M*  $K_2CO_3$ , 85 vol. 0.1 *M*  $NaHCO_3$ ) pH 9.2.

Aliquots of the same cell suspension. Two hundred cu. mm. of cells in each vessel. Gas space in the two vessels with culture medium, 5%  $CO_2$  in air; in the vessel with carbonate buffer, air.

The entire experiment with the same cell suspensions in the sequence reported below.  $\Delta J = 5.4$  for all experiments, except the last, where  $\Delta J$  was 2.75.

Vessel No. 7 (pH 9.2)

7 ml. carbonate buffer

$$v = 13.824$$

$$v_F = 7.00$$

$$k_{O_2} = 0.657$$

Respiration compensated

$$\left. \begin{array}{ll} 5' J & 0 \\ 15' (J + \Delta J) & + 11.5 \\ 15' J & 0 \\ 15' J + \Delta J & + 11.5 \\ 10' J & - 0.5 \end{array} \right\} \begin{array}{l} 30' (J + \Delta J) + 23 \text{ mm.} \\ 30' J \quad \quad \quad - 0.5 \text{ mm.} \\ \hline 30' H \quad \quad \quad + 23.5 \text{ mm.} \end{array}$$

$$y_{O_2} = 23.5 \times 0.657 = 15.4 \text{ cu. mm.}$$

$$\frac{1}{\Phi} - \frac{\Delta J \times 30}{15.4} = 10.5$$

## No. 5 (pH 5)

$$v = 13.913 \quad v_F = 7.00$$

$$k'_{O_2} = 0.665 \quad k'_{CO_2} = 1.253$$

$$15' J + 15.5 \quad 15' (J + \Delta J) + 29.5$$

$$15' J + 17.5 \quad 15' (J + \Delta J) + 29.0$$

$$30' J + 33.0 \quad 30' (J + \Delta J) 58.5$$

$$30' H' + 25.5$$

$$\left. \begin{array}{l} y_{O_2} + 41.3 \text{ cu. mm.} \\ y_{CO_2} - 43.0 \text{ cu. mm.} \end{array} \right\} \gamma = -1.04$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 30}{41.3} = 3.9$$

## No. 7 (pH 9.2)

Respiration not compensated

Carbonate buffer

$$k_{O_2} = 0.657$$

$$\begin{array}{rcl} 10' \text{ dark} & - & 33.5 \\ 10' \Delta J & - & 23.5 \\ 10' \text{ dark} & - & 30.5 \\ 10' \Delta J & - & 22.5 \\ 10' \text{ dark} & - & 30.0 \\ 20' \text{ dark} & - & 60.5 \end{array} \quad \begin{array}{rcl} 20' J & - & 46.0 \\ 20' \text{ dark} & - & 62.7 \\ \hline 20' H & + & 16.7 \end{array}$$

$$y_{O_2} = 16.7 \times 0.657 = 11.0 \text{ cm.}$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 20}{11} = 9.8$$

## No. 5 (pH 5)

$$k'_{O_2} 0.665 \quad k'_{CO_2} 1.253$$

$$10' \text{ dark} - 12 \quad 10' \Delta J - 5.0$$

$$10' \text{ dark} - 13 \quad 10' \Delta J - 4.5$$

$$10' \Delta J - 4.0$$

$$20' \text{ dark} - 25$$

$$30' \text{ dark} - 37.6$$

$$30' H' + 24.1$$

$$\left. \begin{array}{l} y_{O_2} + 45.6 \text{ cu. mm.} \\ y_{CO_2} - 53.0 \text{ cu. mm.} \end{array} \right\} \gamma = -1.18$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 30}{45.6} = 3.6$$

## No. 3 (pH 5)

$$k_{O_2} 1.046 \quad k_{CO_2} 1.634$$

$$10' \text{ dark} - 4.0 \quad 10' \Delta J - 1.5$$

$$10' \text{ dark} - 5.0 \quad 10' \Delta J - 1.5$$

$$10' \text{ dark} - 5.0$$

$$30' \text{ dark} - 14 \quad 20' \Delta J - 3.0$$

$$30' \Delta J - 4.5$$

$$30' H + 9.5$$

No. 7 (pH 9.2)

$$k_{O_2} = 0.657$$

$$\Delta J = 2.25$$

$$10' \Delta J - 24$$

$$10' \text{ dark} - 28 \qquad 30' \Delta J - 71.5$$

$$10' \Delta J - 24.5 \qquad 30' \text{ dark} - 82.5$$

$$10' \text{ dark} - 27.0$$

$$10' \Delta J - 23.0 \qquad 30' H + 11.0$$

$$y_{O_2} = 11 \times 0.657 = 7.2 \text{ cu. mm.}$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 30}{7.2} = \frac{82.5}{7.2} = 11.5$$

The duration of the experiment was about 8 hr. No notable decrease of the efficiency was observed.

In culture medium at pH 5 the quantum requirement was the same below and above the compensation point and was on the average 3.75.

In carbonate buffer at pH 9.2 the quantum requirement was the same below and at the compensation point, and 10.6 as average.

Thus, the quantum requirement for aliquots of the same cell suspension was nearly 3 times greater in carbonate buffer than in culture medium.

### Experiment 3.

June 6, 1949

Culture medium, pH 5. 5% CO<sub>2</sub> in air. 290 cu. mm. cells in each vessel.

$$\Delta J = 6.05$$

No. 5

$$v = 13.913$$

$$v_F = 7.00$$

$$k'_{O_2} = 0.665$$

$$k'_{CO_2} = 1.253$$

$$10' \text{ dark} - 25$$

$$10' \Delta J - 14$$

$$10' \text{ dark} - 22$$

$$10' \Delta J - 13$$

$$10' \text{ dark} - 21.5$$

$$10' \Delta J - 11.5$$

$$30' \text{ dark} - 68.5$$

$$30' \Delta J - 38.5$$

$$30' H' + 30.0$$

No. 3

$$v = 17.993$$

$$v_F = 7.00$$

$$k_{O_2} = 1.046$$

$$k_{CO_2} = 1.634$$

$$10' \text{ dark} - 8.5$$

$$10' \Delta J - 5.5$$

$$10' \text{ dark} - 10.5$$

$$10' \Delta J - 5.0$$

$$10' \text{ dark} - 9.0$$

$$10' \Delta J - 3.0$$

$$30' \text{ dark} - 28.0$$

$$30' \Delta J - 13.5$$

$$30' H + 14.5$$

$$\left. \begin{array}{l} y_{O_2} + 43.4 \text{ cu. mm.} \\ y_{CO_2} - 42.2 \text{ cu. mm.} \end{array} \right\} \gamma = -0.97$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 50}{43.4} = 4.2$$

An aliquot of the same cell suspension was placed in a rectangular vessel with 2 side-arms, to which were added 0.2 ml. of 1 *N* NaOH and filter paper.

Culture medium pH 5, gas space, air.

$$v = 18.477; v_F = 7.40; k_{O_2} = 1.062.$$

290 cu. mm. of cells.

60' dark - 183

60'  $\Delta J$  - 163

60'  $H + 20$  mm.

$$y_{O_2} = 20 \times 1.06 = 21 \text{ cu. mm.}$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 60}{21} = \frac{6.05 \times 60}{21} = 17.3$$

This was one of our first experiments to indicate that light does not act on respiration at intensities otherwise yielding high efficiencies with adequate  $CO_2$  pressure. Later experiments showed that better  $CO_2$  removal by alkali and still less light action was obtained when the filter paper was replaced with glass beads.

#### Experiment 4.

June 10, 1949

Culture medium, pH 5. 5%  $CO_2$  in air. 300 cu. mm. of cells in each vessel.

$$\Delta J = 5.3$$

No. 3		No. 5	
$v = 13.913$	$v_F = 7.00$	$v = 17.993$	$v_F = 7.00$
$k'_{O_2} = 0.665$	$k'_{CO_2} 1.253$	$k_{O_2} = 1.046$	$k_{CO_2} 1.634$
10' dark - 19.5	10' $\Delta J$ - 11.5	10' dark - 9.0	10' $\Delta J$ - 5.5
20' dark - 45.0	20' $\Delta J$ - 26.0	20' dark - 17.0	20' $\Delta J$ - 10.5
30' dark - 59.0	30' $\Delta J$ - 32.5	30' dark - 22.5	30' $\Delta J$ - 13.0
60' dark - 123.5	60' $\Delta J$ - 69.0	60' dark - 48.5	60' $\Delta J$ - 29.0
60' $H' + 54.5$		60' $H + 19.5$	

$$\left. \begin{array}{l} y_{O_2} = +114 \text{ cu. mm.} \\ y_{CO_2} = -140 \text{ cu. mm.} \end{array} \right\} \gamma = -1.23$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 60}{114} = \frac{318}{114} = 2.8$$

Besides the quantum requirement of 3, this experiment shows that the "timing" has no dominant influence on the quantum requirement. For the pressure changes in both vessels were for different time periods:

$$\text{No. 5: } H' = (h'_J + \Delta J + h'_J)$$

$$10' + 8 \text{ mm.}$$

$$20' + 19 \text{ mm.}$$

$$30' + 26.5 \text{ mm.}$$

$$\text{No. 3: } H = (h_J + \Delta J + h_J)$$

$$10' + 3.5 \text{ mm.}$$

$$20' + 6.5 \text{ mm.}$$

$$30' + 9.5 \text{ mm.}$$

The above values are perfectly within the limits of the possible errors.

### Experiment 5.

June 13, 1949

Culture medium, pH 5. 5% CO<sub>2</sub> in air. 300 cm. of cells in each vessel.

$$\Delta J = 5.3$$

No. 5

No. 3

$$v = 13.913$$

$$k'_{O_2} = 0.665$$

$$v_F = 7.00$$

$$k'_{CO_2} = 1.253$$

$$v = 17.993$$

$$k_{O_2} = 1.046$$

$$V_F = 7.00$$

$$k_{CO_2} = 1.634$$

$$10' \text{ dark} - 26.0$$

$$10' \text{ dark} - 27.5$$

$$10' \text{ dark} - 26.5$$

$$10' \text{ dark} - 22.0$$

$$10' \text{ dark} - 21.0$$

$$10' \Delta J - 15.0$$

$$10' \Delta J - 14.5$$

$$10' \Delta J - 15.5$$

$$10' \Delta J - 10.5^*$$

$$10' \Delta J - 15.5$$

$$10' \text{ dark} - 10.5$$

$$10' \text{ dark} - 10.5$$

$$10' \text{ dark} - 11.5$$

$$10' \text{ dark} - 10.5$$

$$10' \text{ dark} - 11.0$$

$$10' \Delta J - 7.0$$

$$10' \Delta J - 7.5$$

$$10' \Delta J - 6.5$$

$$10' \Delta J - 6.5$$

$$10' \Delta J - 7.0$$

$$50' \text{ dark} - 123.0$$

$$50' H' + 52$$

$$50' \Delta J - 71.0$$

$$50' \text{ dark} - 54.0$$

$$50' H + 19.5$$

$$50' \Delta J - 34.5$$

$$\left. \begin{aligned} y_{O_2} &= +104 \text{ cu. mm.} \\ y_{CO_2} &= -130 \text{ cu. mm.} \end{aligned} \right\} \gamma = -1.25$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 50}{104} = 2.5$$

From the dark values in No. 5 and No. 3 the respiration was calculated:

$$50' \text{ dark } H' = -123 \text{ mm.} \quad H = -54 \text{ mm.}$$

$$\left. \begin{aligned} x_{O_2} &= -101 \text{ cu. mm.} \\ x_{CO_2} &= +232 \text{ cu. mm.} \end{aligned} \right\} \gamma = -1.15$$

and  $x_{O_2}$  for 60' - 242 cu. mm. or  $\frac{242}{300} \times 100 = 81\%$  of the cell volume/hr.

This experiment is remarkable for the low average figure of 2.5 obtained in a very good experiment. The only deviating reading among 20 readings is the one marked by \*.

### Experiment 6

June 14, 1949

Culture medium, pH 5. 5% CO<sub>2</sub> in air. 280 cu. mm. of cells in each vessel. Both vessels continuously illuminated with white light for 27 hr.

$$\Delta J = 5.4$$

No. 5

No. 3

$$v = 13.913$$

$$k'_{O_2} = 0.665$$

$$v_F = 7.00$$

$$k'_{CO_2} = 1.253$$

$$v = 17.993$$

$$k_{O_2} = 1.046$$

$$v_F = 7.00$$

$$k_{CO_2} = 1.634$$

## I. Beginning of measurement

June 14, 1:30 p. m.

10' J - 0.5	10' J + $\Delta J$ + 8.0	10' J + 2.0	10' J + $\Delta J$ + 4.5
10' J - 0.5	30' J + $\Delta J$ + 22.5	10' J + 0.5	30' J + $\Delta J$ + 14.0
10' J + 1.5	30' J + $\Delta J$ + 26.0	10' J + 1.0	30' J + $\Delta J$ + 14.5
10' J 0		10' J + 1.5	
		10' J + 1.0	

40' J + 0.5	70' J + $\Delta J$ + 56.5	50' J + 6.0	70' J + $\Delta J$ + 33
70' J + 0.9		70' J + 8.4	
	70' H' + 55.6		70' H + 24.6

$$\left. \begin{array}{l} y_{O_2} + 91 \text{ cu. mm.} \\ y_{CO_2} - 103 \text{ cu. mm.} \end{array} \right\} \gamma = -1.13$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 70}{91} = 4.2$$

## II. Start of 2nd measurement

June 15, 9:40 a. m.

No. 1		No. 3	
10' J + 2.5	10' J + $\Delta J$ + 11	10' J + 2.5	10' J + $\Delta J$ + 7.0
10' J + 3.0	10' J + $\Delta J$ + 12	10' J + 2.0	10' J + $\Delta J$ + 6.5
20' J + 5.5	20' J + $\Delta J$ + 23	20' J + 4.5	20' J + $\Delta J$ + 13.5
	20' H' + 17.5		20' H + 9.0

$$\left. \begin{array}{l} y_{O_2} + 22.4 \text{ cu. mm.} \\ y_{CO_2} - 20.2 \text{ cu. mm.} \end{array} \right\} \gamma = -0.9$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 20}{22.4} = 4.8$$

## III. Start of 3rd measurement

June 15, 4:50 p. m.

The cells were washed in fresh culture medium because in the old medium the efficiency of the light appeared to decrease.

No. 5		No. 3	
10' dark - 17.5	10' $\Delta J$ - 10.0	10' dark - 8.5	10' $\Delta J$ - 6.0
10' dark - 16.0	10' $\Delta J$ - 8.5	10' dark - 8.5	10' $\Delta J$ - 5.0
10' dark - 16.5	10' $\Delta J$ - 7.5	10' dark - 8.5	10' $\Delta J$ - 6.5
10' dark - 15.5			10' $\Delta J$ - 5.5
40' dark - 65.5	30' $\Delta J$ - 26.0	30' dark - 25.5	40' $\Delta J$ - 23.0
30' dark - 49.2			30' $\Delta J$ - 17.2
	30' H' + 23.2		30' H + 8.3

$$\left. \begin{array}{l} y_{O_2} = +48.5 \text{ cu. mm.} \\ y_{CO_2} = -62 \text{ cu. mm.} \end{array} \right\} \gamma = 1.28$$

$$\frac{1}{\Phi} = \frac{\Delta J \times 30}{48.5} = 3.4$$

The respiration at the beginning of the experiment was 82% of the cell volume  $O_2$ /hr., and at the end of the experiment 45% of the cell volume  $O_2$ /hr. The washing of the cells in fresh culture medium had no influence on the respiration.

The experiment shows that the efficiency of light remained nearly constant when the cells were continuously illuminated with white light for more than 20 hr.

*Experiment 7.*

June 16, 1949

5%  $CO_2$  in air. The cells were washed with  $KNO_3$ -free culture medium (pH 5) and the efficiency was measured in  $KNO_3$ -free culture medium. Later  $KNO_3$  was added and the efficiency was again measured. 300 cu. mm. of cells in each vessel.

No. 5		$\Delta J = 5.4$	No. 3	
$v = 13.913$	$v_F = 7.00$		$v = 17.993$	$v_F = 7.00$
$k'_{O_2} = 0.665$	$k'_{CO_2} = 1.253$		$k_{O_2} = 1.046$	$k_{CO_2} = 1.634$
without $KNO_3$				
10' dark - 26	10' $\Delta J$ - 17		10' dark - 12	10' $\Delta J$ - 7
10' dark - 23	10' $\Delta J$ - 15		10' dark - 10	10' $\Delta J$ - 7
10' dark - 23	10' $\Delta J$ - 14		10' dark - 11	10' $\Delta J$ - 7
10' dark - 23	10' $\Delta J$ - 15		10' dark - 11	10' $\Delta J$ - 7
40' dark - 95	40' $\Delta J$ - 61		40' dark - 44	40' $\Delta J$ - 28
40' $H' + 34$			40' $H + 16$	
$\left. \begin{aligned} y_{O_2} &= -51.6 \text{ cu. mm.} \\ y_{CO_2} &= -53.7 \text{ cu. mm.} \end{aligned} \right\} \gamma = -1.04$				
$\frac{1}{\Phi} - \frac{\Delta J \times 40}{51.6} = 4.2$				

Then 17.5 mg.  $KNO_3$  was added to each vessel, to give 2.5 g.  $KNO_3$ /l.

No. 5		No. 3	
10' dark - 17	10' $\Delta J$ - 9	10' dark - 8	10' $\Delta J$ - 5
10' dark - 15	10' $\Delta J$ - 9	10' dark - 7	10' $\Delta J$ - 5
10' dark - 16.5	10' $\Delta J$ - 8.5	10' dark - 7.5	10' $\Delta J$ - 5.5
30' dark - 48.5	30' $\Delta J$ - 26.5	30' dark - 22.5	30' $\Delta J$ - 15.5
30' $H' + 22$		30' $H + 7.0$	
$y_{O_2} = + 50$ cu. mm. $y_{CO_2} = - 66.7$ cu. mm.		$\gamma = - 1.33$	
$\frac{1}{\Phi} = \frac{\Delta J \times 30}{50} = 3.2.$			

This experiment shows that there is no striking effect of  $KNO_3$  in the medium on the photosynthetic efficiency. In very prolonged experiments, of course, removal of a necessary constituent from the culture medium must influence the efficiency.

*Experiment 8.*

July 20, 1949, Woods Hole

5% CO<sub>2</sub> in air. Culture medium, pH 5.

430 cu. mm. of cells in each vessel.

$$\Delta J = 5.65$$

No. 5		No. 3	
$v = 13.913$	$v_F = 7.00$	$v = 17.993$	$v_F = 7.00$
$k'_{O_2} = 0.665$	$k'_{CO_2} = 1.253$	$k_{O_2} = 1.046$	$k_{CO_2} = 1.634$
Compensated with white light from above.			
$10' J + 29.5$	$10' J + \Delta J + 40.5$	$10' J + 19.5$	$10' J + \Delta J + 26$
$10' J + 26.0$	$10' J + \Delta J + 39.0$	$10' J + 20.0$	$10' J + \Delta J + 24.5$
<hr/>	<hr/>	<hr/>	<hr/>
$20' J + 55.5$	$20' J + \Delta J + 79.5$	$20' J + 39.5$	$20' J + \Delta J + 50.5$
$20' H' + 24$		$20' H + 11$	
$\left. \begin{array}{l} y_{O_2} = + 37.2 \text{ cu. mm.} \\ y_{CO_2} = - 41.2 \text{ cu. mm.} \end{array} \right\} \gamma = - 1.10$			
$\frac{1}{\Phi} = \frac{\Delta J \times 20}{37.2} = 3.0$			

Continued, but compensated mainly from below with light from an incandescent lamp filtered through a red glass which transmitted wavelengths longer than 560 mμ.

No. 5		No. 3	
$5' J + 18$	$5' J' + \Delta J + 22.5$	$10' J' + 19$	$10' J + \Delta J' + 24$
$5' J + 16.5$	$5' J' + \Delta J + 25.0$	$10' J' + 16$	$10' J + \Delta J' + 22$
$5' J + 16.0$	$5' J' + \Delta J + 20.0$		
<hr/>	<hr/>	<hr/>	<hr/>
$15' J + 50.5$	$15' J' + \Delta J + 67.5$	$20' J' + 35$	$20' J + \Delta J + 46$
$20' H' + 22.7$		$20' J + 11$	
$\left. \begin{array}{l} y_{O_2} = + 32.3 \text{ cu. mm.} \\ y_{CO_2} = - 33.0 \text{ cu. mm.} \end{array} \right\} \gamma = - 1.02$			
$\frac{1}{\Phi} - \frac{\Delta J \times 20}{32.3} = 3.5$			

Finally all light was turned off:

No. 5	No. 3
$10' \text{ dark} - 11$	$10' \text{ dark} - 6$
$10' x_{O_2} = - 12.4 \text{ cu. mm.}$	
$60' x_{O_2} = - 74 \text{ cu. mm.,}$	

that is,  $\frac{74}{420} \times 100 = 18\%$  of the cell volume/hr.

At  $J' + \Delta J$  in vessel 5, + 45 mm. were produced in 10'. The light action was therefore  $45 + 12 = 57$  mm. in 10'. Assuming a value of  $\gamma = 1.0$ , this means  $y_{O_2} = 57 \times 1.42 \times 6 = 485$  cu. mm. O<sub>2</sub>/hr., or  $485/74 = 6.5$  times respiration.

This experiment shows that 1) the efficiency of the added red light was essentially the same whether the respiration was overcompensated from above or from below, with white light or with red-yellow light; 2) a quantum requirement of 3.5 was obtained when respiration was 6.5 times compensated with a red-yellow light and a value of 3.0 when respiration was similarly overcompensated with white light.

*Experiment 9.*

August 5, 1949, Woods Hole

Culture medium, pH 5. In vessels 5 and 3, 5% CO<sub>2</sub> in air. In vessel 34 air, in the two side-arms NaOH. In each vessel 230 cu. mm. of cells, suspended in 7 ml. culture medium.

$$\Delta J = 5.7$$

No. 5		No. 3	
$v = 13.913$	$v_F = 7.00$	$v = 17.993$	$v_F 7.00$
$k'_{O_2} = 0.665$	$k'_{CO_2} = 1.253$	$k_{O_2} = 1.046$	$k_{CO_2} 1.634$

Respiration not compensated

20' dark - 27.5	20' $\Delta J$ - 7	20' dark - 13	20' $\Delta J$ - 2		
20' $H' + 20.5$ mm.		$H + 11$ mm.			
$y_{O_2} = + 23.6$ cu. mm.		$\gamma = - 0.82$			
$y_{CO_2} = - 19.3$ cu. mm.					
$\frac{1}{\Phi} = \frac{\Delta J \times 20}{23.6} = 4.8$					

Respiration overcompensated

No. 5		No. 3			
20' $J + 57.5$	20' $J + \Delta J + 76$	20' $J + 39$	20' $J + \Delta J + 48$		
20' $H' + 18.5$		20' $H + 9$			
$y_{O_2} = + 26$ cu. mm.		$\gamma = - 1.0$			
$y_{CO_2} = - 26$ cu. mm.					
$\frac{1}{\Phi} = \frac{\Delta J \times 20}{26} = 4.4$					

No. 34		
$v = 18.87$	$v_F = 7.40$	$k_{O_2} = 1.09$
10' dark - 22	} no light action	
10' $\Delta J$ - 21		
10' dark - 18		
10' $\Delta J$ - 18		
$\frac{1}{\Phi} = \frac{\Delta J \times t}{y_{O_2}} = > 100$		

But when a 100-w. incandescent lamp was turned on at a distance of 12 in. from vessel 34, the respiration was compensated by the white light.

This experiment shows, that at adequately low  $\text{CO}_2$  pressure there was no light action by  $\Delta J$ .

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#### SUMMARY AND CONCLUSIONS

Experiment No. 2 shows that the quantum requirement in carbonate buffer at pH 9 was 2-3 times the quantum requirement in acid culture medium saturated with 5%  $\text{CO}_2$  in air (pH 5). This result was obtained for aliquots of the same cell suspension. It invalidates many efficiency experiments of the past, where for 10 years the conventional medium for quantum requirement determinations has been carbonate buffer, and the view has been widely adhered to that the minimum quantum requirement could be realized in carbonate buffer. But in no experiment in carbonate buffer at pH 9 have we observed lower quantum values than 8, the average being about 10.

Experiment No. 9 shows that in acid culture medium the quantum requirement is different according as the medium is saturated with 5%  $\text{CO}_2$  or freed as nearly as possible of  $\text{CO}_2$ . When the culture medium was largely freed of  $\text{CO}_2$ , no  $\text{O}_2$  was produced by a light intensity that in the presence of 5%  $\text{CO}_2$  was highly efficient.

In all experiments carried out by the prescribed procedure in acid culture medium at pH 5, and saturated with 5%  $\text{CO}_2$  in air, low quantum requirements were observed. The results of Expts. Nos. 1 to 9 are summarized in the following table in sequence. The asterisks mean that the respiration was overcompensated. In the doubly asterisked experiments the respiration was more than 6-fold compensated.

It may be seen from Table I that in acid culture medium saturated with 5%  $\text{CO}_2$  in air the quantum requirements per molecule of  $\text{O}_2$  produced were between 2.5 and 4.8, on the average near to 4. The reality of 3 in several instances cannot be excluded. Four-quanta in the

red at 660 m $\mu$  mean 65% efficiency and 3-quanta would mean 87% efficiency in the transformation of light to chemical energy.

The observed assimilatory quotients,  $\gamma = \text{CO}_2 \text{ absorbed} / \text{O}_2 \text{ produced}$ , ranged between  $-0.8$  and  $-1.33$ , with an average of  $-1.06$ . The deviation from  $-1.0$  was in the opposite direction from that claimed by investigators who have reported outbursts of  $\text{CO}_2$  in light (4,5). On the contrary, on the average a little more  $\text{CO}_2$  was absorbed in light than  $\text{O}_2$  was produced. Thus, one of the two main loopholes that have

TABLE I

$\frac{1}{\Phi}$ $\left(\frac{\text{h}\nu}{\text{O}_2}\right)$	$\gamma$ $\left(\frac{\text{CO}_2}{\text{O}_2}\right)$
4.6	-0.80
2.9*	-1.03*
4.5*	-0.90*
3.9*	-1.04*
3.6	-1.18
4.2	-0.97
2.8	-1.23
2.5	-1.25
4.2*	-1.13
4.8*	-0.90*
3.4	-1.28
4.2	-1.04
3.2	-1.33
3.0**	-1.10**
3.5**	-1.02**
4.8	-0.82
4.4	-1.00*
Average 3.7	-1.06

been used to evade the high efficiency in photosynthesis is now closed. The other important loophole—the participation of respiration in efficient photosynthesis in whatsoever a manner (14)—is now closed too, since the same high efficiencies have been obtained at more than 6-fold compensated respiration as at uncompensated respiration, where light has also been shown directly to be without notable influence.

The fact must thus be envisaged that in a perfect nature photosynthesis is perfect too.

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## The Determination of Polyunsaturated Fatty Acids in Blood <sup>1</sup>

In a study of methods for the quantitative determination of polyunsaturated fatty acids in small volumes of blood, we have investigated the possibility of adapting the method of alkali isomerization (1, 2, 3) to the analysis of milligram quantities of blood lipides.

A total lipide extract was prepared from 1 ml. of beef plasma according to the general procedure recommended by Kirk, Page and Van Slyke (4). The petroleum ether extract was transferred to a 8" × 10" Pyrex test tube. The solvent was evaporated and the lipides were saponified in 2 ml. of methanolic potassium hydroxide by warming for 90 min. at 60°C. in an atmosphere of nitrogen. This preliminary saponification was carried out in consideration of the findings reported by Front and Daubert (5) in the determination of linoleic acid in cholesteryl linoleate. The alkali isomerization was performed as described by Front and Daubert (5), employing 5 ml. of alkaline ethylene glycol for each sample and a total heating period of 25 min. The sample and the appropriate blank were transferred with absolute methanol to 100-ml. volumetric flasks and further diluted, when necessary, to optical densities suitable for measurement in the Beckman spectrophotometer. The ultraviolet absorption spectrum of such a preparation is shown in Fig. 1.

The spectrum is characteristic of an alkali isomerized lipide sample containing conjugated dienoic, trienoic, and tetraenoic acids (maxima at 233, 268, and 315 m $\mu$ , respectively). The maximum at 345 m $\mu$  has been interpreted by Holman and Burr (6) as an indication of the presence of pentaene acids. The initial studies reported here have been confined to measurements at 233 m $\mu$ .

In order to determine the reproducibility of the procedure, four 1-ml. aliquots of calf plasma were extracted, and the lipides were saponified

<sup>1</sup> A preliminary note.

and isomerized, and the spectrophotometric measurement was carried out, as described above. The final solution volume was 100 ml. The optical density at 233  $m\mu$  averaged .581, with a mean deviation of .009. A similar experiment was performed using 1-ml. aliquots of beef plasma. The average optical density of 6 samples was .577, with a mean deviation of .006.

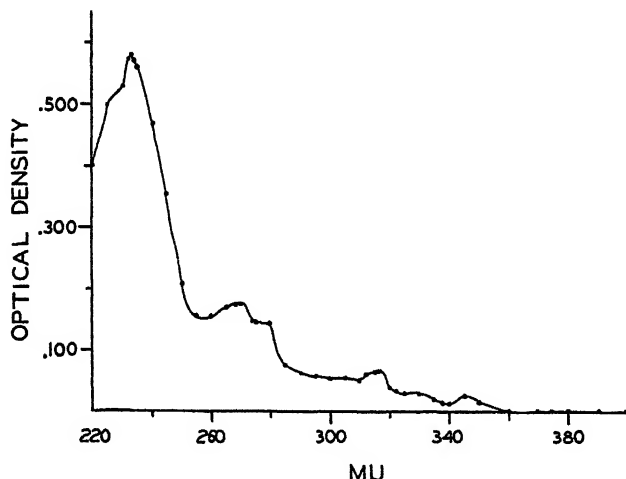


FIG. 1. Ultraviolet absorption spectrum of alkali isomerized lipides from 1 ml. of beef plasma. Final volume: 100 ml. Solvent: absolute methanol.

A sample of methyl linoleate having an  $E_{1cm}^{1\%}$  value of 850 was dissolved in beef plasma to yield a concentration of 33.6 mg.-% of added ester. One-ml. volumes of this plasma sample were then extracted and analyzed together with 1-ml. volumes of the stock plasma. The concentration of the added ester was calculated by means of the following formula:

$$\text{Mg.-% methyl linoleate} = \frac{D_E - D_S}{.85} \times 100.$$

Where  $D_E$  = Optical density at 233  $m\mu$  of sample containing added ester. (Calculated for final volume of 100 ml.)

$D_S$  = Optical density of stock plasma sample at 233  $m\mu$ . (Final volume 100 ml.)

.85 =  $E_{1cm}^{1\text{mg.}\%}$ .

100 is the dilution factor of the sample.

Table I gives the result of this experiment.

The method of calculation employed in this experiment permits the estimation of the concentration of the added ester, but, although the intensity of absorption at 233  $m\mu$  in the stock sample is proportional to the concentration of isomerized linoleic acid derived from its various lipide compounds, the true plasma concentration of the acid cannot be determined until the contribution of two additional factors has been assessed. There is a general absorption due to all the lipide components ("Background Absorption") and a further contribution due to diene conjugation of acids more unsaturated than linoleic (linolenic, arachidonic, etc.)

TABLE I  
*Analysis of Plasma for Methyl Linoleate*

Mg.-% of added ester	Per cent recovery
33.4	99.4
33.1	98.5
32.1	95.5
33.3	99.1

The background absorption may be estimated by a measurement of the absorption of the plasma lipides when dissolved in a suitable solvent such as isooctane. In general this absorption amounts to 10–15% of that observed following the alkali isomerization. Preliminary results indicate that linolenic acid is not present in plasma lipides. The concentration of arachidonic acid, however, may be sufficient to account for as much as 25% of the total absorption at 233  $m\mu$ . Work now in progress is aimed at extending the measurements into the tetraene region in order to estimate the concentration of arachidonic acid and, thus, to facilitate the calculation of the linoleic acid concentration.

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## Studies on Nitrogen Trichloride-Treated Prolamines.

### VI. Suppression of the Development of Convulsions with Methionine

On the basis of elementary analysis and molecular-weight determination the empirical formula of  $C_5H_{12}N_2O_3S$  was derived for the neurotoxic factor which we recently isolated in a crystalline, chromatographically homogeneous form from nitrogen trichloride-treated zein (1). It was also shown that the compound is not a peptide since the alpha-amino nitrogen content did not increase upon prolonged hydrolysis. It contains neither  $-SH$ ,  $-SS-$  nor an acidic group similar in strength to a sulfonic acid group. These findings and the fact that it was formed from a sulfur-containing protein suggested the possibility that it might be a derivative of methionine. Furthermore, it appeared to be possible that the substance exerts its toxic effect through antagonism with methionine. It was thus of interest to study the effect of methionine on the convulsions caused by the toxic factor.

Male albino rabbits (New Zealand-Chinchilla breed) weighing about 1 kg. and male albino mice ( $CF_1$ ) weighing about 20 g. were used. (We found recently that mice are also susceptible to this toxic factor.) Crystalline substances isolated from various batches and toxic concentrates were given orally, intraperitoneally, and intravenously. The intensity of symptoms and the onset of convulsions was independent of the route of administration. The substance to be tested for its effect on the toxicity was given to rabbits orally in three equal doses 5 hr. before, simultaneously, and 24 hr. after the administration of the toxic factor unless otherwise stated. To mice it was given intraperitoneally half an hour before the intravenous injection of the toxic factor. The results of typical experiments are given in Table I.

Depending on the amount of toxic material used, methionine either suppressed the convulsions or delayed their onset. The inhibition of toxicity by methionine could be overcome, however, by increasing the dose of the toxic material in relation to the dose of methionine. Under the experimental conditions given, the ratio of the dose of toxic substance to the minimum inhibiting dose of methionine was about 1:300 in rabbits and 1:50 or less in mice.

Heathcote (2) reported that the toxic factor inhibits the growth of *Leuconostoc mesenteroides*. Newell and Carman in our laboratories found that this growth inhibition is also reversed by methionine (3).

Methionine sulfone, methionine sulfoxide, homocystine, cystine, creatinine, ethanolamine and  $\alpha$ -aminobutyric acid were also tested for inhibitory effects. Only homocystine showed some inhibitory effect manifested in a delay of the onset but not in a suppression of the

TABLE I  
*Effect of Methionine on Convulsions*

Toxic material		Methionine dose, mg./kg.	Convulsions		
Designation	Dose, mg./kg.		Number of animals		
			Not having	Having	Onset, hr.
Rabbits					
Conc. No. 194	300 <sup>a</sup>	—	—	3	Within 24
Conc. No. 194	300	500 <sup>b</sup>	—	1	In 48
Conc. No. 194	300	1500 <sup>c</sup>	2	—	—
Conc. No. 194	450	1500 <sup>c</sup>	1	—	—
Conc. No. 194	600	1500 <sup>c</sup>	—	1	In 48
Cryst. No. 132	4 <sup>a</sup>	—	1 <sup>d</sup>	3	Within 48
Cryst. No. 132	4 <sup>a</sup>	1500 <sup>c</sup>	4	—	—
Cryst. No. 258	2.5 <sup>a</sup>	—	—	1	Within 72
Cryst. No. 258	5 <sup>a</sup>	—	—	2	Within 24
Cryst. No. 258	5 <sup>a</sup>	1500 <sup>c</sup>	2	—	—
Cryst. No. 258	10 <sup>a</sup>	1500 <sup>c</sup>	1	1	In 24
Mice					
Cryst. No. 132	40 <sup>a</sup>	—	—	4	In 7
Cryst. No. 132	40 <sup>a</sup>	2500 <sup>f</sup>	4	—	—

<sup>a</sup> Orally; <sup>b</sup> simultaneously with the toxic material orally; <sup>c</sup> in three divided doses orally; <sup>d</sup> in a near convulsive stage; <sup>e</sup> intravenously; <sup>f</sup> intraperitoneally.

convulsions. With all other substances convulsions occurred at about the same time as in their controls.

Attempts to influence with methionine convulsions caused by metrazol and nikethamide have been unsuccessful so far.

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## Growth of *Polyporus versicolor* in a Medium with Lignin as the Sole Carbon Source<sup>1</sup>

Evidence for the decomposition of lignin by certain fungi has been demonstrated by various investigators (1, 2, 3). However, the growth of these fungi in chemically defined media containing isolated native lignin has not been reported. It has been demonstrated (4) that some of the white rot fungi produce a definite growth response to an isolated lignin incorporated in a synthetic medium composed of inorganic salts, asparagine, and thiamine. It was necessary to use an adaptation technique to achieve this growth response and asparagine was a necessary component of the basal medium. Recently it has been found that *Polyporus versicolor* is capable of growth in a medium without asparagine, lignin being the sole source of carbon. Moreover, no adaptation period is required; growth occurs in the chemically defined basal medium supplemented with lignin in a period of 1-2 weeks.

## EXPERIMENTAL

The composition of the basal medium employed was as follows:  $\text{NH}_4\text{NO}_3$ , 2.5 g.;  $\text{KH}_2\text{PO}_4$ , 1.5 g.;  $\text{K}_2\text{HPO}_4$ , 1.5 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g.;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (trace); and thiamine, 1 mg., all dissolved in 1000 ml. of water. The resulting pH was  $6.5 \pm .1$ .

The technique employed for measurement of growth response to lignin was as described previously (4). Ten ml. of double strength basal medium was dispensed in 125-ml. Erlenmeyer flasks and sterilized by autoclaving. To this sterilized medium was added 10 ml. of a 1% aqueous suspension of sterile (autoclaved) native lignin. The lignin employed was prepared according to the method described by Brauns (5). The resulting flasks were inoculated with a piece of mycelium approximately 0.5 cm. square taken from a mat grown on potato-dextrose agar. Simultaneously, flasks

<sup>1</sup> This work was done under contract No. N7-onr-397-4 between the University of Maryland and the Office of Naval Research. The project was initiated at the suggestion of the Prevention of Deterioration Center, National Research Council.

containing basal medium plus glucose and unsupplemented basal media were inoculated. Results are presented in Table I.

The results presented in Table I are indicative of the clear-cut growth response obtained with lignin as the sole carbon source in the synthetic medium. Growth, as evidenced by diameter of mat, was almost equal to that obtained with a glucose medium. Since none of the three strains of *Polyporus versicolor* tested required previous adaptation to a lignin medium, it is concluded that this behavior is characteristic of this species, in contrast to that of the organisms previously reported (4).

TABLE I

*Growth Response of Three Strains of Polyporus versicolor after 7 Days' Incubation on a Chemically Defined Basal Medium Supplemented with Lignin or Glucose*

Culture designation <sup>a</sup>	Composition of media					
	Basal		Basal plus lignin (0.5%)		Basal plus glucose (0.5%)	
	Growth response					
	Mold mat	Final pH	Mold mat <sup>b</sup>	Final pH	Mold mat <sup>b</sup>	Final pH
37	—	6.4	5.0	5.1	5.5	3.9
37A	—	6.4	6.0	4.0	6.0	3.1
37B	—	6.4	5.0	4.4	5.5	3.0

<sup>a</sup> Culture 37 received from W. G. Campbell, Forest Products Research Laboratories, Princes Risborough, England.

Cultures 37A and 37B received from Ross W. Davidson, Bureau of Plant Industry, Beltsville, Maryland.

<sup>b</sup> Diameter of mold mat in centimeters.

This is the first demonstration of a fungus being cultivated in a synthetic liquid medium in which the sole carbon source is represented by isolated native lignin. Significant, too, is the fact that growth takes place at a comparatively rapid rate, good growth occurring from 7–14 days, in contrast to the longer periods which are generally reported for growth on wood and other lignin-containing media.

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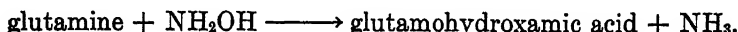
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## Observations on a Plant Amide Enzyme System Requiring Manganese and Phosphate

An enzyme system has been isolated from sugar pumpkin seedlings that specifically catalyzes the exchange reaction:



The stoichiometry of the reaction was established experimentally by the demonstration that in the formation of 2.9  $\mu$ moles of hydroxamic acid, 2.8  $\mu$ moles of  $\text{NH}_3$  were simultaneously liberated.

The enzyme is stable in water solution, can be stored at  $-10^\circ$ , is completely destroyed on exposure for 5 min. at  $60^\circ$ , and is completely inhibited by fluoride in a final concentration of  $10^{-3}$  *M*. In contrast to the glutamine synthesizing system of Speck (1), this system is not inhibited by iodoacetic acid and does not require -SH reagents for activation. Asparagine is inactive in the system.

As indicated in Table I, manganese (Mn) is a component of the enzyme system. The following cations in a final concentration of  $10^{-3}$  *M* have been found ineffective in replacing Mn:  $\text{Mg}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Cu}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Be}^{++}$ ,  $\text{Ba}^{++}$ , and  $\text{Al}^{+++}$ . The  $K_m$  value for Mn has been estimated to be  $2.5 \times 10^{-5}$  *M*.

The second component can be either (1) inorganic phosphate, or (2) arsenate. Coenzyme A, adenosinetriphosphoric acid (ATP) and adenosinediphosphoric acid (ADP) are ineffective in the system. Arsenate is 2 to 3 times as effective as phosphate. It is most effective when present in amounts equivalent to the initial concentration of glutamine. However, because of the presence of phosphatases in the system no clear-cut statement can be made as to the cyclic nature of phosphate or arsenate in the system. No accumulation of  $\gamma$ -glutamyl phosphate or arsenate could be detected in enzyme mixtures in

absence of  $\text{NH}_2\text{OH}$  as a trapping agent. Experiments are now being devised to overcome these difficulties.

In analyzing the effects of ammonia and amino acids on the formation of glutamylhydroxamic acid from glutamine and  $\text{NH}_2\text{OH}$ , it was consistently observed that a tenfold concentration of  $\text{NH}_3$  above that of the initial glutamine concentration completely inhibited hydroxamic acid formation. Similar results were obtained with glycine, alanine, aspartic acid, and serine whereas 11 other amino acids gave little or no inhibition. The cause of this inhibition is now under investigation.

TABLE I

*Enzyme System*

The enzyme system was prepared by extracting 1 g. of acetone powder of pumpkin seedlings with 20 ml. of water, filtering through cheese cloth, and adjusting the pH of the extract to 5.5 with 1% acetic acid. The precipitate was discarded and the supernate was adjusted to a pH of 4.8. The resulting precipitate was dissolved in 10 ml. of 0.1%  $\text{NaHCO}_3$ . The complete system contained 0.5 ml. of enzyme, 0.5 ml. of 0.1 *M* tris (hydroxymethyl) amino methane buffer at pH 7.2, 0.1 ml. of 0.01 *M* manganese sulfate, 0.1 ml. of 0.1 *M* hydroxylamine (freshly neutralized), and 0.1 ml. of 0.1 *M* arsenate of pH 7.2. System incubated for 60 min. at 34° and analyzed for hydroxamic acid by the method of Lipmann and Tuttle (3). Hydroxamic acid expressed in terms of succinohydroxamic acid used as standard.

System	Hydroxamic acid formed <i>μ</i> moles
Complete	3.1
Complete without arsenate	0.1
Complete without Mn	0.0
Complete without arsenate; phosphate added	1.05
Complete + fluoride, $10^{-3}$ <i>M</i> final conc.	0.0

The observation of the high degree of specificity of Mn in this system may be correlated with the recent interesting observations of Hewitt *et al.* (2) who in analyzing the amino acid composition of plants grown in Mn-deficient medium reported large accumulations of amino acids as well as asparagine and glutamine and therefore concluded that the absence of Mn disrupted the normal amino acid metabolism of plants.

In addition to this system, crude enzyme extracts of pumpkin seedlings have been found to form in the presence of  $\text{NH}_2\text{OH}$  hydroxamic acids from aspartic acid, glutamic acid, and asparagine. These findings are now under further study.

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### Effect of Vitamin B<sub>12</sub> on the Response to Homocystine in Chicks

Chicks were hatched from eggs laid by hens on a diet containing no source of "animal protein factor" (1). The chicks (Barred Rock × New Hampshire) were markedly deficient in vitamin B<sub>12</sub>. They were fed a diet deficient in methionine and B<sub>12</sub>, and the effects of various supplements were studied.

The basal diet was as follows: Glucose ("Cerelese") 62.5 g.; soybean protein,<sup>1</sup> alcohol-extracted, 25 g.; calcium gluconate, 5 g.; bone ash, 2 g.; salt mixture (2), 2 g.; L-cystine, 0.3 g.; dimethylaminoethanol HCl, 0.2 g.; corn oil plus vitamins A, D, and E (2), 3 g.; inositol, 0.1 g.; calcium pantothenate, 5 mg.; niacinamide, 5 mg.; riboflavin, 1 mg.; pyridoxine HCl, 1 mg.; thiamine HCl, 1 mg.; vitamin K compound (2), 0.5 mg.; folic acid, 0.2 mg.; biotin, 0.02 mg. After a depletion period of 4 days on the basal diet, the following supplements were fed per kilo of diet: Groups 1 and 5, 6 g. DL-methionine; groups 2 and 6, 6 g. DL-homocystine; groups 3 and 7, 6 g. homocystine plus 1 g. betaine hydrochloride; groups 4 and 8, no supplement. Groups 1 to 4 received vitamin B<sub>12</sub>, 2 μg. injected weekly per chick, while groups 5 to 8 did not receive B<sub>12</sub>. Seven chicks were used in each group, and the results are shown in Fig. 1.

Homocystine,<sup>2</sup> with or without betaine, did not promote the growth of vitamin-B<sub>12</sub>-deficient chicks on a diet deficient in methionine. However, these chicks responded to the addition of methionine. When supplemented with B<sub>12</sub>, the chicks responded to methionine, homocystine, or homocystine *plus* betaine.

<sup>1</sup> Archer Daniels Midland Company, Minneapolis, Minnesota.

<sup>2</sup> Homocystine was kindly supplied by Drs. V. du Vigneaud and John Wilson.

A relation between "animal protein factor" and the methionine requirement of chicks was indicated by the results of Patton and co-workers (3) who noted that the growth-promoting effect of methionine as a supplement to a corn-soybean diet was no longer observed when 2% of sardine meal was added to the basal diet. Cunha and co-workers (4) found that methionine improved the growth of pigs on a corn-peanut-meal diet only in the absence of an animal protein factor supplement.

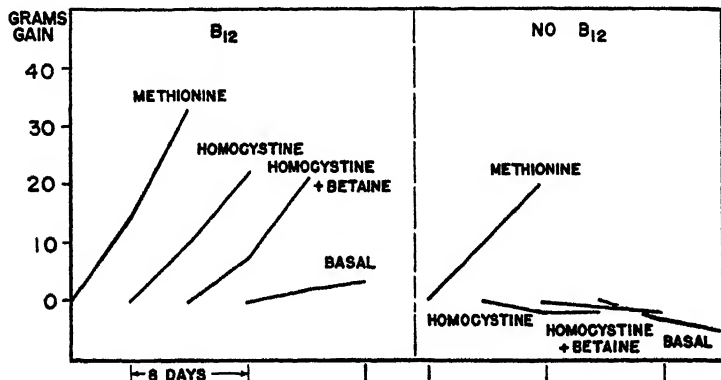


Fig. 1. Growth curves on chicks, groups 1 to 8 inclusive reading from left to right, receiving a basal diet deficient in  $B_{12}$  and methionine together with various supplements. Each curve represents an 8-day period.

It was found by Shive and co-workers (5) that methionine and vitamin  $B_{12}$  could function interchangeably in enabling growth of *Escherichia coli* to take place on a medium containing sulfanilamide. The present investigation indicates that vitamin  $B_{12}$  may be needed for the transformation of homocystine to methionine in chicks. Other functions for vitamin  $B_{12}$  in the nutrition of chicks are indicated by the observation that growth was more rapid on methionine plus  $B_{12}$  than on methionine alone, and by the finding (1) that vitamin  $B_{12}$  is needed for growth and survival of chicks on a diet of natural foods supplemented with methionine and choline.

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## Effect of Animal Protein Factor on Lowering Protein Needs of the Pig

Using a corn-peanut meal ration Cunha *et al.* (1) showed that Animal Protein Factor (APF) (Lederle) and vitamin B<sub>12</sub> were different in their response on the pig. With the same corn-peanut meal ration Burnside *et al.* (2) showed that APF (Lederle) increased the feeding value of peanut meal and soybean oil so that these plant protein supplements were similar in feeding value to the fish meal used. Again with the same corn-peanut meal ration, Cunha *et al.* (3) found that APF (Lederle) and B<sub>12</sub> spared the methionine needs of the pig. These later studies also indicated that APF (Lederle) contains B<sub>12</sub> plus some other factor. These data are in agreement with those of Stokstad *et al.* (4) who found that the same APF supplement supplied some unidentified factor in addition to B<sub>12</sub> for the chick. The control ration used in this trial consisted of corn, peanut meal, bone meal 0.5, limestone 0.5, and salt 0.5%. The corn and peanut meal content of the various rations was varied to give rations containing levels of 19.6, 17.9, 15.9, and 12.2% total protein as shown in Table I. Vitamins A and D, thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, choline, and folic acid were added to the rations in lots 1 to 8 at the same levels used in the previous study by Cunha *et al.* (3). Only vitamins A and D, at the same levels used in lots 1 to 8, were added to the rations in lots 9 and 10. Five purebred Duroc pigs were fed in each lot on concrete floors. The floors were washed once daily. The trial lasted for 6 weeks during a period when the protein requirements of growing (weaned) pigs are at their highest.

The data in Table I show that the pigs fed the 15.9 and 17.9% protein rations + APF gained at a faster rate than the pigs fed the 19.6% protein ration without APF. The pigs fed the 12.2% protein ration plus APF gained as rapidly as the pigs fed the 19.6% protein ration without APF. These data show that the APF supplement is of considerable benefit in affecting protein requirements of the pig when the ration lacks the factors contained in the Lederle APF concentrate.

Thus even on a low protein diet comparatively good growth was obtained when the APF supplement was added.

The 19.6% protein ration without APF was slightly superior to the 17.9% protein ration without APF; however, when APF was added to these comparable rations then the rate of gain was the same at the 17.9 or 19.6% protein level. This shows that the protein level in the ration can be lowered to approximately 18% by adding APF under the conditions of this experiment.

TABLE I  
Summary of Data

Lot no.	Av. starting wt.	Level of protein in ration	Av. daily gain each week						Av. daily gain during 6 weeks	Hb. Avg. <sup>c</sup>
			1st	2nd	3rd	4th	5th	6th		
1	18.6	19.6	0.56	0.66	0.93	0.97	1.0	0.63	0.79	11.4
2	19.0	19.6+APF <sup>a</sup>	1.00	0.90	1.20	1.34	1.45	1.36	1.21	12.9
3	18.1	17.9	0.61	0.59	0.56	0.86	0.89	0.80	0.72	10.5
4	19.4	17.9+APF	0.99	1.03	1.16	1.29	1.60	1.20	1.21	12.3
5	18.4	15.9	0.51	0.56	0.67	0.66	0.86	0.60	0.64	10.4
6	18.5	15.9+APF	0.91	0.84	1.30	1.26	0.94	0.63	0.98	11.2
7	19.1	12.2	0.44	0.14	0.51	0.47	0.51	0.36	0.41	9.4
8	19.3	12.2+APF	0.69	0.70	0.79	0.67	1.06	0.93	0.81	10.6
9	19.5	19.6 <sup>b</sup>	0.57	0.50	0.66	0.64	0.89	0.59	0.64	10.7
10	19.0	19.6+APF <sup>b</sup>	1.03	1.00	1.31	1.26	1.44	1.21	1.21	12.8

<sup>a</sup> One thousand g.\* added to 100 lb. of feed in all lots. APF Supplement (N203B and 8108-09) obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, New York. The APF Supplement was dried *S. aureofaciens*, Fraction I, which was shown by Stokstad *et al.* (4) to contain B<sub>12</sub> plus some factor as yet unidentified for the chick.

<sup>b</sup> The 7 B-complex vitamins were not included in lots 9 and 10.

<sup>c</sup> Average of hemoglobin levels at end of the experiment.

The rate of gain obtained at the 19.6% protein level (lot 1) to which the B-complex vitamins were added to the ration was greater than at the 19.6% protein level (lot 9) where the B-complex vitamins were omitted. These data show that some of the vitamins added to the ration were beneficial. A comparison of the data obtained in lot 2 (19.6% protein with B-vitamins) and lot 10 (19.6% protein + APF without B-vitamins) shows that the APF supplement had a beneficial effect in stimulating growth even though the B-vitamins were not added to the ration. However, it must be kept in mind that the APF

supplement contains a certain amount of B-vitamins (5). Of interest is the fact that the pigs in lot 9 on 19.6% protein without B-vitamins did not even gain as fast as the pigs fed 12.2% protein + APF + B-vitamins.

The Lederle APF supplement, as in previous trials (1, 2, 3), increased bloom and caused a smoother hair coat in the pigs.

The hemoglobin levels show a trend very similar to the data obtained on the growth rate. The tendency seems to be for a slight increase in hemoglobin level where APF is fed. This is very consistent in all lots.

These data indicate that efficient utilization of protein by pigs under the conditions of our experiments was obtained only when the diet contained the APF supplement. These data indicate that the accepted values for the protein requirements of swine may need to be re-evaluated by using adequate amounts of vitamin B<sub>12</sub>, plus other factors present in the Lederle APF supplement, in the ration.

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### The Bio-Oxygenation of Steroids at C-11<sup>1</sup>

We have previously reported that isolated perfused adrenal glands transform 11-desoxycorticosterone (DOC) or 11-desoxycorticosterone acetate (DOCA) into corticosterone (I)(1). At the present time we wish

<sup>1</sup> The work described in this paper was supported by a grant from G. D. Searle and Company.

to report that the biochemical introduction of the 11( $\beta$ )-hydroxyl function into the steroid nucleus is not specific for DOC but also occurs with 17( $\alpha$ )-hydroxy-11-desoxycorticosterone-21-acetate (17-OH DOCA) and androsterone.

As in the previous work (1), steroids dissolved in propylene glycol were added to homologous citrated blood or plasma and perfused, with a pulsatile pressure, through cow adrenal glands. In the initial studies, the steroids were recovered from perfusates by prolonged dialysis of the perfusate against large (10–50) volumes of sodium chloride–sodium citrate solution, followed by  $\text{CHCl}_3$  or  $\text{CH}_2\text{Cl}_2$  extraction of the dialysate. When it was found that corticosteroids could be adsorbed from aqueous solution by charcoal (Darco G-60) and eluted without alteration by  $\text{CHCl}_3$  or  $\text{CH}_2\text{Cl}_2$ , steroids were removed from perfusates by dialysis against an equal volume of saline-citrate containing suspended charcoal and, later, by direct charcoal treatment of plasma and blood (hemolyzed by freezing and thawing). After elution from charcoal, the residues thus obtained were fractionated by chromatography on silica gel (Davison T-200).

From adrenal perfusates wherein 17-OH DOCA was employed, 17( $\alpha$ )-hydroxy corticosterone (II), m. p. 200–5°,<sup>2</sup> was isolated as a crystalline transformation product. The identity was established by the melting point, 200–5°, of a mixture with an authentic sample of II (Upjohn), m. p. 206–8°, and by a demonstration that the products obtained from perfusates and authentic II have identical infrared absorption spectra in the frequency region from 800 to 1180  $\text{cm}^{-1}$ . As expected from the melting point, the infrared curve of the perfusate product indicated the presence of small amounts of unknown contaminants.

From a similar perfusate of androsterone, there was isolated an amorphous fraction which, after acetylation, was crystallized and identified as 11( $\beta$ )-hydroxyandrosterone 3-acetate, m. p. 238–40°. Its mixture with an authentic sample of 11( $\beta$ )-hydroxyandrosterone 3-acetate, m. p. 243–5° (obtained from Dr. R. Dorfman) melted at 240–5°. The infrared absorption curves of the acetates were identical.<sup>3</sup>

<sup>2</sup> All melting points were taken on a Fisher-Johns block and are uncorrected.

<sup>3</sup> The infrared analysis of perfusate products was performed by Dr. K. Dobriner, Sloan-Kettering Institute, N. Y., whose generous cooperation is hereby acknowledged.

The above data thus satisfactorily demonstrate that androsterone is converted into 11( $\beta$ )-hydroxyandrosterone (**III**).

The view that **I**, **II**, and **III** represent transformation products of the steroid substrates introduced now seems conclusively established since:

(a) Perfusion of glands, with either blood or plasma without added steroids or adrenocorticotrophic hormone, did not give rise to detectable amounts of crystalline **I**, **II**, or **III**.

(b) In 20 experiments with DOC only **I** was found and never **II** or **III**; in 3 experiments with 17-OH DOCA only **II** was found; and finally in 5 experiments using a variety of steroids, other than the three steroid substrates described, **I**, **II**, or **III** was never obtained.

(c) The amounts of **I** and **II** obtained by adrenal perfusion are greater than the amounts of these compounds present in the gland. Thus in a representative experiment with DOC, in which 605 mg. of DOC was perfused through a gland weighing 12.5 g., 81.5 mg. of crystalline **I** and 173 mg. of crystalline DOC were isolated from the perfusate. When 190 mg. of 17-OH DOCA was perfused through a gland weighing 12.1 g., 9.5 mg. of crystalline **II** and 28.5 mg. of 17-OH DOC were obtained. Thus, 17-OH DOCA is deacetylated during the course of perfusion or in the work-up procedure, as had previously been observed with DOCA (1).

From isolation studies on beef adrenals (cf. 2), it may be calculated that a 12.0 g. adrenal should contain less than 0.1 mg. **I** or **II**.

(d) **III** is a 3( $\alpha$ )-hydroxy steroid which has not been isolated from adrenal tissue. The 3( $\beta$ )-hydroxy isomer of **III**, which has been found in human urine, has been generally regarded as an excretion product of 11-oxycorticosteroid metabolism, a view which merits re-examination in the light of the present work.

In initial perfusion experiments employing 4-androstene-3, 17-dione and progesterone, suggestive evidence for 11( $\beta$ )-hydroxylation of both of these steroids has been found, but further work is necessary to establish conclusively the structures of the transformation products.

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## Enzymatic Resolution of Racemic $\beta$ -2-Thienylalanine<sup>1</sup>

It has been shown in this laboratory that  $\beta$ -2-thienylalanine may be conveniently synthesized from thiophene by a combination of the *N*-methylformanilide (1) and rhodanine syntheses (2). The purpose of this communication is to report the successful enzymatic resolution of this unnatural racemic amino acid into its optical isomers through the asymmetric hydrolysis of the *N*-chloroacetylated derivative by means of carboxypeptidase (3).

### EXPERIMENTAL

Chloroacetyl- $\beta$ -2-thienyl-DL-alanine was prepared by the method outlined for the chloroacetylation of L-phenylalanine (4). To verify the identity of this compound, it was converted to the azlactone by treatment with acetic anhydride and pyridine (5). When mixed with an authentic sample obtained from 2-thenaldehyde by means of the Erlenmeyer azlactone synthesis, no depression in melting point was observed. The carboxypeptidase was prepared from fresh-frozen beef pancreas (3) and stored at  $-14^{\circ}\text{C}$ . as a suspension in water.

### Enzymatic Resolution

The procedure (6) used for the resolution of chloroacetyl-DL-phenylalanine was modified in this work. The major improvements consisted in the addition of a MacIlvaine buffer of pH 7.6 to the digest and the use of 1 *N* HBr rather than 2 *N* HCl for the chemical hydrolysis of the chloroacetylated D-isomer. Hydrolysis with 2 *N* HCl and subsequent evaporations to dryness *in vacuo* 3 times caused partial racemization; an optically pure D-amino acid was obtained when 1 *N* HBr was utilized, with one evaporation.

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<sup>1</sup> This study was carried out under the auspices of the Office of Naval Research with the cooperation of J. V. Fiore and S. N. Timasheff of this laboratory.

TABLE I  
*Specific Rotations of Optical Isomers of  $\beta$ -2-Thienylalanine*

Compound	$[\alpha]_D^{21^\circ}$ Observed		$[\alpha]_D^{21^\circ}$ Literature	
	L-form	D-form	L-form	D-form
$\beta$ -2-Thienylalanine	-31.4 <sup>a</sup>	+31.4 <sup>b</sup>	-31.7 (7)	+31.6 (7)
Chloroacetyl- $\beta$ -2-thienyl- alanine	+46.5 <sup>c</sup>	-47.2 <sup>d</sup>		

<sup>a</sup> 1.0834 g. in 50 ml. of water.

<sup>b</sup> 0.2624 g. in 25 ml. of water.

<sup>c</sup> 0.8468 g. in 50 ml. of absolute alcohol.

<sup>d</sup> 0.9487 g. in 50 ml. of absolute alcohol.

The specific rotations of the optical isomers obtained are presented in Table I.

Full details of this investigation with analytical data will be presented in due course.

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## Book Reviews

**Advances in Enzymology. Vol. 9.** Edited by F. F. NORD. Interscience Publishers, Inc., New York, 1949. x + 760 pp. Price \$9.00.

This ninth volume of the *Advances in Enzymology* is larger than its predecessors. In addition to reviews on various sections of enzymology, it contains some original articles of fundamental importance. One of these is the introductory article of L. Michaelis on reversible step reactions. Michaelis combines here his theory on one-step oxidoreduction with his classical theory of an enzyme-substrate complex, and advances a general theory on the dissociation of compounds of the type  $AB_n$  into  $A + n B$ . Criteria are advanced which permit decision of whether the combination of A with a ligand B alters the affinity of A for other ligands of the type B. If there is no change in the affinity of A for B, a fundamental dissociation constant can be calculated by means of statistical methods. In oxidoreductions the electron plays the role of B.

The article of A. E. Stearn on the "Kinetics of Biological Reactions" supplements previous articles on thermodynamics by the same author. The article deals chiefly with the heat denaturation of proteins and enzymes. The thermodynamic data lead to the surprising result that the energy content of the denatured protein is higher than that of the native protein. Since the spontaneous occurrence of denaturation indicates a decrease in the free energy content, the increase in total energy content is ascribed to an increase in entropy. Unfortunately, we are not yet able to interpret unequivocally the thermodynamic data. The reviewer wonders, for this reason, whether one is entitled to calculate activation energies for the "chirping of crickets" or the "flashing of fireflies." Such figures do not indicate more than the well-known statement that the rate of biological reactions increases 2- or 3-fold if the temperature rises by  $10^{\circ}\text{C}$ . This criticism does not affect, however, the stimulating and lucid presentation of the complicated problems by Stearn.

The first two articles are followed by a series of reviews on particular fields of enzymology, each of them accompanied by 300-400 references. A. D. McLaren discusses the action of light on the physicochemical properties of proteins and enzymes. M. A. Lauffer, W. C. Price and A. W. Peters report on the present state of the virus problem. John Runnström, to whom we owe most of our knowledge on the processes accompanying fertilization of the sea-urchin egg, reports on fertilization in metazoa, and T. Mann on the metabolism of semen. Both articles emphasize the great importance of hyaluronidase or a similar liquefying enzyme in the jelly coat of the egg and also in semen.

The volume contains also a short chapter by D. Glick on cyto- and histochemical methods, a discussion of the nitrogen metabolism in higher plants by H. E. Street, a review on nucleic acids by F. Schlenk, and 3 articles on problems of industrial biochemistry and enzymology. T. K. Walker treats of the formation of citric acid and other organic acids in molds, A. Hesse the synthesis of fats by molds, and M. A. Joslyn the influence of freezing and scalding of tissues on their enzymatic activity.

The reviewer was particularly impressed by the article of F. Schlenk, because the large amount of material was not only well arranged, but also discussed critically; by such a criticism only can we learn the limits of our knowledge and the points to be attacked by further research.

From this description of the last volume of the *Advances in Enzymology* it is evident that it contains a number of outstanding contributions and that, therefore, it is indispensable for enzymologists and other biochemists. The editor and the publishers are to be congratulated for publishing this very valuable volume.

F. HAUROWITZ, Bloomington, Indiana

**Laboratory Experiments in Biological Chemistry.** By JAMES B. SUMNER, Professor of Biochemistry, Cornell University, and G. FRED SOMERS, Associate Professor of Biochemistry, Cornell University. Academic Press, Inc., New York, N. Y., 1949. viii + 167 pp. Price \$3.50.

The first 14 pp. of this laboratory book are devoted to general instructions concerning the use of reagents, laboratory rules, cleaning of glassware and other similar problems. This part of the book will be particularly useful for students of biology or bacteriology who are not as well acquainted with laboratory usages as students of chemistry.

The laboratory experiments are arranged in two parts. Part I (67 pp.) treats of lipides and carbohydrates, and also the analysis of blood, urine, and gastric juice. Amino acids, proteins, and enzymes are discussed in the second part. Obviously, a laboratory book contains only a selection of experiments, dictated by the tradition and the equipment of the laboratory in which they are carried out. No wonder that experiments on enzymes occupy a very large part of a book whose coauthor is Professor Sumner, and that the preparation of the common proteins is replaced by the preparation of crystalline urease and other jack bean proteins. This makes the book valuable for advanced students interested in enzymology.

The description of the experiments is very clear, the style short and lucid, the print of the book excellent. Throughout the book the tendency prevails to prevent thoughtless mechanical work, and to induce the student to think over the analytical problems. The book is recommended particularly to those who wish to obtain authoritative and reliable information on the preparation of enzymes.

F. HAUROWITZ, Bloomington, Indiana

**Radioactive Measurements with Nuclear Emulsions.** By HERMAN YAGODA, Senior Physical Chemist, National Institutes of Health. John Wiley and Sons, Inc., New York; Chapman and Hall, Ltd., London, 1949. 356 pp. Price \$5.00.

One of the most important methods of investigating radioactive radiations is by means of the ionization which they produce in passing through a photographic emulsion. Many of the great advances, both in pure and applied radioactivity, have been made using simple detectors such as the photographic plate and the scintillation screen. Notable among these are Becquerel's fundamental discovery of the radioactive radiation emitted by uranium and Rutherford's detection of the protons from the first discovered transmutation processes. More recently, photographic emulsions

have been applied in recording the first artificially created mesons. Unlike the cloud chamber, the photographic emulsion records continuously and presents an integrated picture; this makes it invaluable in the study of rare radioactive events. The importance of this tool has been further enhanced by the availability in recent years of thick, fine-grained emulsions of exceptionally high AgBr content, which were designed primarily to record heavily ionizing radiation. Since physicists, chemists, mineralogists and biologists are equally interested in the application of photographic methods to radioactive studies, the appearance of a comprehensive volume dealing with the various aspects of radioactive measurements with nuclear emulsions will be warmly welcomed by workers in all these fields.

The book has been designed in the first place as a guide on the use of emulsions in radioactive measurements, but sufficient theoretical material has been incorporated to enable the reader to understand the basic mechanism underlying the working methods. A wealth of useful information has been collected by the author, his book covering a much broader field than its title would lead one to expect. For example, in connection with the demonstration of track formations produced by radiocolloids, starting from the description of Paneth's discovery of these colloids, a comprehensive review of radiocolloid aggregation is given. A short preface is followed by the following chapters: Photographic detection of nuclear particles. Comparison of scintillation and photographic methods. Laboratory manipulations.  $\alpha$ -particle patterns of nuclear emulsions. Quantitative aspects of the  $\alpha$ -particle pattern. Radiochemical studies with nuclear emulsions.  $\alpha$ -Particle patterns of uranium and thorium minerals.  $\alpha$  Tracers in crystallography and metallurgy. Biological applications of  $\alpha$ -particle tracers. Principles of  $\beta$ -particle autoradiography. Applications of  $\beta$ -ray patterns. Applications in nuclear physics. Appendix 1. Range energy relations in Ilford nuclear research emulsions. Appendix 2. Atomic constants and conversion factors.

The book is illustrated by a great number of beautiful photographs, demonstrating such widely differing phenomena as  $\alpha$ -stars from a thorium emulsion, localization of radio-iodine in unstained tissue, autoradiographs of mineral sections activated by neutron bombardment, and a track of a negative meson which produces a nuclear evaporation.

This comprehensive and excellent book is warmly recommended to all those interested in pure or applied radioactivity, and especially to those interested in the application of photographic methods to such studies.

G. HEVESY, Stockholm, Sweden

**Acids, Bases and Non-Aqueous Systems.** by LUDWIG F. AUDRIETH, Professor of Inorganic Chemistry, University of Illinois, Urbana, Ill. Sponsored by Phi Lambda Upsilon and the Department of Chemistry, Pennsylvania State College, State College Pa., 1949. vii + 66 pp. Price \$2.00.

These, the 23rd Annual Priestley Lectures, were given April 25-29, 1949. The small lithoprinted volume consists of a one-page Foreword and 5 chapters, dealing, respectively, with "The Historical Development of the Acid-Base Concept and Its Philosophical Aspects," "Modern Concepts," "The Nitrogen System of Compounds," "The Hydronitrogens," and "Acid-Base Relationships in High Temperature Systems."

The historical development of the acid-base-salt theories is briefly traced from Gerber through the iatrochemical period, the Renaissance and the phlogistonists, to the dawn of modern chemistry, to the Arrhenian and Werner theories, and finally to the modern Brönsted-Lowry and Lewis theories. The second chapter begins with the work of Walden, Cady, Franklin and Kraus, comparing the water and ammonia systems, stressing the Franklin concept of aquo and ammonio compounds and expanding the idea to include protonic and non-protonic solvents. From the solvent system the Brönsted-Lowry formulation, based on proton donors and acceptors, is developed, and finally the Lewis electronic system, based on electron pair donors and acceptors, leading first to the formation of covalent bonds.

In Chapter III, ammoniation, ammonolysis, and deammoniation in liquid ammonia, followed by solvolytic reactions in general are discussed. From these the aquo-, ammonio-, and mixed aquo-ammonio-compounds of C, S, and P are considered.

Chapter IV develops the direction of specific attention to the chemistry of nitrogen. From consideration of ammonia, hydrazine and hydrazoic acid, and their organic derivatives, came the development of the saturated and unsaturated hydronitrogens and the consideration of these substances as ammonio compounds. In this section are considered also the reduction products of nitric and hydrazoic acids, the hydrazine system of compounds and the heterocyclic carbonic acid derivatives of ammonia.

Chapter V considers the acid-base relationship in fusion mixtures, particularly from the standpoint of the Lewis theory, although the Brönsted-Lowry theory is also discussed.

Considering the general excellence, the lucid and logical presentation, and the obvious importance of the subject, together with its academic and industrial applications, it is really too bad that the proof-reading of the text was not carried out more carefully. This is annoying to the reader at times. For example (p. iii, Foreword, l. 3), Calvinism is not "Calvanism," (p. 2, 3rd line of last paragraph) "the" should be "that," (p. 4, p. 8) "to" should be "too," (p. 8, 3rd line from bottom) "to" should read "in," (p. 10, 8th line from bottom) "face" should be "fact," (p. 28, ref. 8) author's name is "Emel  us," (p. 31, l. 20) "Guanidine," (p. 36, l. 2) "nitridotrisulfuric," (p. 45, Fig. V. legend) "phosphoric," (p. 41, ref. 2) insert "of" after "series," (p. 45, l. 7) "2-tetrazenes," (p. 48, l. 7) "noble" (heading, Table V) "Hydrazoic," (p. 55, Table VII) bonds omitted in two formulae, (p. 59, l. 7, 1st paragraph) "attacked," (p. 64, l. 3) "polymeta-." There are many cases of improper punctuation, particularly lack of punctuation, which, in one or two cases, make it necessary to reread passages to determine the authors' meaning. There are also many cases of improper hyphenation, both omission and commission, and a few cases of improper spacing and alignment. These, however, are faults of printing and can be remedied when reprinting becomes necessary.

W. A. HYNES, New York, N. Y.

**Chemical Activities of Fungi.** By JACKSON W. FOSTER, Professor of Bacteriology, University of Texas, Austin, Texas. Academic Press Inc., New York, N. Y., 1949. xviii + 648 pp., with illustrations. Price \$9.50.

It is undoubtedly true that interest in the subject of mold metabolism has increased enormously during the last decade; reference to penicillin alone may suffice

as an explanation. However, as clearly set forth in the book under review, the chemical activities of fungi have already been exploited in several other ways as well. In addition, it appears that there is still ample room for extension of these practical applications. Dr. Foster emphasizes in this respect that in present industrial processes carbohydrates are mostly used as the substrate, and he shows that the use of different substrates may lead to the formation of new and unexpected products.

Moreover, the number of fungal species which have been subjected to a thorough metabolic investigation is very limited as compared with the total number of fungi known. The fact that the catalog of the large collection of fungi present in the "Centraalbureau voor Schimmelcultures" at Baarn (Netherlands) mentions several thousand different species, all available in pure culture, opens the possibility for a quick start in the further exploration of this field.

It can be safely predicted that Dr. Foster's admirably presented and well-balanced survey of the state of our knowledge regarding mold metabolism will act as a strong stimulus upon future research work.

It should, however, be well understood that the book offers much more than a mere compilation of the widely scattered data present in literature. As stated in the Preface, the book owes its origin to the bewilderment which overcame the author when as a graduate student in 1936 he was assigned by his teacher, Dr. S. A. Waksman, to a problem in mold metabolism. Even then he was already impressed by the enormous amount of work accomplished—albeit of a highly contradictory nature—and at the same time by the unavailability of any suitable treatise dealing with the subject.

From this moment on, Dr. Foster keenly felt the need of an authoritative and critical book integrating and evaluating the field. It required, however, another ten years before he could familiarize himself with the idea that the prodigious task of writing such a book was up to himself. As things are we can only rejoice at this delay. In the first place, war conditions led Dr. Foster to become one of the pioneers in the field of penicillin production, and hereby he must have gained a wide experience in mastering tricky problems in mold metabolism. Nevertheless, to the reviewer it seems even more important that in the period preceding the war Dr. Foster had availed himself of the opportunity to widen his views considerably by applying himself to quite remote subjects in the field of general microbiology. The names of his microbiological mentors, Drs. Waksman, Starkey and van Niel, to whom the book has been dedicated, guarantee the thoroughness of his initiation in this wider field. As a result thereof, numerous pages in the book testify to the fact that this book has not been written merely by a specialist in mold physiology, but by one well versed in the general principles of microbiology. For this reason the book, far from being only useful to beginning students, will also render most valuable services to experienced workers in the mold domain.

In order to give a good idea of the broad way in which Dr. Foster has conceived his task the titles of the 19 chapters in which the book has been divided are given here.

Chapter I, Introduction, History and Perspective; II, The Methodology of Mold Metabolism; III, Chemical Nature of the Mold Mycelium; IV, General Consideration on Mold Metabolism; V, Natural Variation; VI, Mutations, Physiological Genetics and Biochemical Syntheses; VII, Trace Element Nutrition of Fungi; VIII, Lactic Acid Formation by Fungi; IX, Alcoholic Fermentation by Molds; X, Oxalic

Acid Metabolism; XI, Fumaric and Other C<sub>4</sub>-Dicarboxylic Acids; XII, Citric Acid; XIII, Itaconic Acid; XIV, Kojic Acid; XV, Gluconic and Other Sugar Acids; XVI, Carbohydrates Produced by Fungi; XVII, Nitrogen Metabolism of Fungi; XVIII, Other Transformations in Fungi; XIX, Microbiological Aspects of Penicillin.

After all this praise it seems only fair to warn the prospective reader that amongst the experiences after the digestion of the book there will also be an element of disappointment. For all Dr. Foster's erudition and cleverness cannot do away with the conclusion that in several cases the metabolic pathways of molds are still almost completely in darkness. Only a wider application of isotopes in metabolic studies than has been possible until now seems to open the possibility for further progress in this direction.

Under present conditions it was unavoidable that the author in several cases had to have recourse to the term "strain specificity" when confronted with contradictory results; nevertheless it seems important to realize that there is the danger that this term will become another veil of our ignorance.

With a view to the diversity of the subjects discussed by the author it is not surprising that Dr. Foster's critical attitude is not always maintained at the same high level. Thus the pages dealing with the energetics of mold metabolism just briefly surveys the earlier work by Terroine and Wurmser and the later studies by Tamiya; here a critical comment would have been most welcome.

The book has been well edited; only a few typographical errors have been noticed. However, it is somewhat confusing that the numbering of Tables and Figures is not continuous for the book as a whole, but starts afresh in each chapter.

It will be clear that these minor remarks are not intended to depreciate in the least the value of the book. To the contrary, the reviewer has the greatest admiration for this remarkable achievement, and it seems to him that anybody interested in microbiological biochemistry owes a debt of profound gratitude to the author—and judging from the Preface also to Mrs. Foster—for the sacrifices made in the preparation of this work of love.

A. J. KLUYVER, DELFT, HOLLAND

**Advances in Carbohydrate Chemistry.** Edited by W. W. PIGMAN, University of Alabama, Birmingham, Alabama, and W. L. WOLFROM, The Ohio State University, Columbus, Ohio. Academic Press Inc., New York, N. Y., 1949. iv + 375 pp. Price \$7.80.

The new fourth volume of this valuable collection brings ten essays of fourteen authors.

The first essay discusses thoroughly *The Structure and Configuration of Sucrose* written by Irving Leve and Clifford B. Purves. The article gives a clear and impressive picture of the difficulties of this problem, of the great amount of work and the many workers who were necessary to master these difficulties up to the high probability of our knowledge today. In the last chapter, the biochemical synthesis of sucrose, this important success in sucrose chemistry, is described.

In the second essay, H. G. Bray and M. Stacey write about *Blood Group Polysaccharides*. It is the nature of the subject that this paper deals especially with the biochemical side. But our chemical knowledges about these polysaccharides, gained

in very laborious work, is described, too, at the proper place. The article will be extremely valuable for everyone who is interested in the chemistry of blood group substances, not only of its carbohydrates.

The third article, by C. S. Hudson, brings a description of *Aprose and the Glycosides of the Parsley Plant*. The author is one of the most successful pioneers in carbohydrate chemistry. One need hardly remark that this "novel" about one of the strangest sugars with its branched structure is a particularly attractive pleasure for every epicure in chemistry, instructive for all who are working with natural substances.

The next article is written by Carl Neuberg about *Biochemical Reductions at the Expense of Sugars*, a field in which we have to thank to a considerable extent this author and his collaborators. It is astonishing for those who are not aware of these reactions in their entirety, how many substances can be reduced by living yeast and other bacteria. The article is valuable, since it invites the use of this reducing method in other cases, too, for scientific and for preparative purpose.

*The Acetylated Nitrile of Aldonic Acids and their Degradation* are the contents of the next article by Venancio Deulofeu. The clearly written essay is an interesting example of degradation reactions in carbohydrate chemistry and gives valuable suggestions on related fields too.

A treatise about *Wood Saccharifications*, its scientific and technological development is given in Article 6 by Elwin E. Harris. The author brings not only a complete summary but also many technological details of this process which may yet be of economic value in the future.

In the next article, J. Boeseken gives an excellent complete and critical summary dealing with his own important work in *The Use of Boric Acid for the Determination of the Configuration of Carbohydrates*. Successes as well as the limits of this method are discussed thoroughly. This essay will help in the use of this unique method in further suitable instances, successfully and without error.

A summary of the chemistry of *The Hexitols and Some of Their Derivatives*, the first of this field, is written by Rolland Lohmar and R. M. Goepp, Jr. The scientific information is extended and amplified by considerable technological data and suggestions.

*Plant Gums and Mucilages* is treated by J. K. N. Jones and F. Smith. These substances, highly interesting in their physical properties and because of their physiological importance present a very difficult task for exact chemical research. This resumé of our present knowledge will help further experiments with these substances, widely distributed in nature.

The last article is dedicated to *The Utilization of Sucrose*, by I. F. Wiggins. This is an economic problem in normal times and may be still more urgent in the future. This survey is valuable in letting us know what has been done, and valuable as offering suggestions as to what may be done further, especially in converting sucrose into other products by fermentation processes.

All the articles give numerous literature references and several very complete tables of the substances in question.

This fourth volume, like its predecessors, is a successful collection, welcomed by chemists and biologists who are interested in the broad field of carbohydrate chemistry, technology, and biology.

B. HELFERICH, Bonn, Germany

**Photosynthesis in Plants.** A monograph of the American Society of Plant physiologists. Edited by JAMES FRANCK, Professor of Chemistry, University of Chicago, and WALTER E. LOOMIS, Professor of Plant Physiology, Iowa State College. The Iowa State College Press, Ames, Iowa, 1949. viii + 500 pp. Price \$7.00.

The preparation of the present volume was initiated at the Gibson Island conference on photosynthesis in 1941. In 1947, Dr. Daniels agreed to publish in the planned monograph the papers read on the A. A. A. S. meeting on photosynthesis which, in most cases, could be arranged.

This information is helpful in understanding the composition of the book. It would have been useful if the origin of each paper had been mentioned and the date of receipt indicated.

A wide variety of subjects is treated in the 22 chapters, giving an impressive manifestation of the amount and quality of the work American investigators have contributed to the understanding of photosynthesis during the last 1 or 2 decades. A balanced treatment of the whole field is not aimed at; some contributors gave profound reviews with numerous references, others communicated a special research problem with few recent references, papers not much different from those to be found in journals. A few are concerned with comparison of opposing views. The chief value of the book lies in its presentation of a vivid picture of present-day problems and interpretations; as such, it is addressed particularly to specialists, although the variety of subjects is sufficiently great to obtain a picture not too far from the complete one.

The introduction by Loomis gives an idea of the field with allusions to subjects and views presented in subsequent chapters. In line with the character of the book, views are presented in this place that are not generally accepted, *e. g.*, that each transferred hydrogen atom requires 2 quantum steps. Also, the figure on p. 15 represents a specialized view. The term "lay plant physiologist" (p. 16) for those investigating external influences on photosynthesis, while not definitely specialized, does not seem advisable.

Thomas and Hill (Chapter 2) chiefly review work of their own, revealing an admirable mastery of the technical difficulties encountered in work under field conditions. The statement that, at low  $\text{CO}_2$  concentration, light reactions may govern the rate of photosynthesis, whereas, at high concentrations, dark reactions control, is surprising. J. H. C. Smith (Chapter 3) gives a profound review on the products of photosynthesis which is suggestive in showing how much we still have to learn about possible paths and bypaths. Verduin (Chapter 4), in a special approach to a special subject: diffusion through multiperforate septa, seeks a theoretical evaluation of the interference between neighboring pores. The renewed consideration of this subject is to be enjoyed in view of its importance for the study of photosynthesis in higher plants.

Granick and Strain review chloroplast structure, composition, and development, and the chloroplast pigments (Chapters 5 and 6). In the statement: ". . . in seed plants, chlorophyll formation depends upon exposure. . . to light" (Strain, p. 134), the term "Angiosperms" would seem preferable. In: "Autotrophic plants and chemoautotrophic bacteria each contain one or two chlorophylls. . ." (p. 141), the term "chemo-autotrophic bacteria" includes also non-photosynthetic ones, but not the *Athiorhodaceae*. In Strain's account on fluorescence of cells and chloroplasts (p. 166) some statements have not sufficiently general acceptance to be compiled in a brief

section without comment. Livingston (Chapter 7) reports on special investigations *in vitro* on fluorescence, photobleaching and related phenomena, with chlorophyll. He finds no support for the belief that photosynthesis and fluorescence in the chloroplasts are complementary. However, the strong influence of the protein bearer upon the properties of chlorophylls in the chloroplasts may not be overlooked. Rothe-mund (Chapter 8) reports on special researches in porphyrin syntheses, with some general considerations. J. H. C. Smith (Chapter 9) reports on the rôle of dark processes in greening of etiolated seedlings.

Chapters 10-13 contain an extensive discussion of quantum efficiency, based on work with algae, manometric, polarographic and calorimetric methods being used. The results confirm an efficiency of 0.08-0.12 mol  $O_2$  or  $CO_2$ /quantum. The recent results of Warburg were not corroborated. Moore and Duggar conclude that no appreciable change in respiratory rate took place during illumination in their experiments. The results of Kok have not yet been considered. Rieke concludes that photosynthesis and photoreduction in algae differ in the way of disposing of the oxidative photobyp-products. In photosynthesis the oxidizing product oxidizes water to  $O_2$ , in photoreduction it oxidizes  $H_2$  to water. This supports the belief—also held by the reviewer—that, in green plant photosynthesis, water is not directly decomposed photocatalytically but enters into a dark reaction like the hydrogen donor in purple bacteria photoreduction. Similarly, Holt and French (Chapter 14) state "the involvement of a Blackman reaction" (p. 278) in the oxygen-liberating photoreactions of chloroplast suspensions.

Chapters 15 and 16 are concerned with the relation between chlorophyll fluorescence and photosynthesis. Katz presents the views of the Utrecht-Delft group about 1941 which, in essence, have not changed. He supplements the facts by a more recent theoretical picture of energy transfer. Later work of the Utrecht-Delft group on diatoms and green algae, throwing some interesting flashes upon the variety of connections between fluorescence and photosynthesis, has not been mentioned. Neither is it considered by Franck, which is surprising, since a large part of his paper is concerned with a detailed reinterpretation of findings by the reviewer and his collaborators. Franck holds that the results on purple bacteria of the Utrecht-Delft group can be understood—as well as many others—by postulating that metabolically formed "narcotics" are responsible for changes in the rate of photosynthesis and the fluorescence yield.—Metabolites as enzyme inhibitors or energy acceptors may well play a rôle during the still too slightly understood induction period of photosynthesis. But to advance this as an explanation also for phenomena in the stationary state would imply a certain underappreciation of the production of "limiting factors" by way of enzyme kinetics. Both viewpoints could be reconciled if all products which are not removed quickly were termed "narcotics," which, however, would hardly clarify the situation. The results on diatoms referred to above oppose Franck's summary-statement that "a rise of the fluorescence yield is always connected with a limitation of the photosynthetic activity."

Myers' work on nitrogen metabolism and photosynthesis (Chapter 17) is attractive in showing that, even in photosynthesis, nowadays attacked with the latest and most refined weapons, common sense and sound reasoning are still apt to yield fundamental results with simple techniques. Chapters 18-21, inclusive, are concerned with tracer-

research. Kamen reviews the work by the late Dr. Ruben and his associates, using  $C^{14}$ , with some recent extensions. Benson, Calvin *c.s.*, report on the work of the Berkeley group; Brown, Fager and Gaffron on the work of the Chicago group. Rather large discrepancies still exist between the views held by these groups. Whereas Calvin's group advocates the idea that a reducing power is generated in the light, while the path of carbon is much like a reversal of respiration, Gaffron's group claims the formation of a specialized C-intermediate by a light reaction.—Curiously, there are independent supports for both views. Evidence for the generation of reducing power by light was derived by the reviewer and his collaborators many years ago from observations on fluorescence of *Chlorella*. Evidence for a photo-product different from those easily available to respiration is found in Myers' experiments on assimilation of glucose by algae, simultaneously with photosynthesis.—The title of Chapter 20: "Kinetics of a Photochemical Intermediate" seems open to linguistic objections.

The book concludes with a treatise on the comparative biochemistry of photosynthesis, by C. B. van Niel. The expectation of an admirable mastery of the subject is not disappointed. In connection with redox potentials, and with the relation  $CO_2$ :  $H_2$  in purple bacteria, work of the Utrecht-Delft group might have been mentioned.

In the title of the book the words "in Plants" seem somewhat superfluous, so far no serious indications for photosynthesis being met with beyond the plant kingdom.

The test shows very few printing failures; some more appear in the reference lists. The majority of these are due to lack of care in quoting authors' names. The spelling of Engelmann's name, especially, should be noted since it concerns one of the great classics in our field! The illustrations are somewhat poor, and more uniformity in magnitude of letters, and quality of the design might be desirable.

The book's modernity is reflected in the reference lists; of the some 800 references only a little over 100 refer to papers which appeared prior to 1931. It is a worthy presentation of the present-day concept of photosynthesis. Its critical study can be warmly recommended to all workers in the field. In addition to the knowledge gained they will find hints as to problems still to be solved.

E. C. WASSINK, Wageningen, Netherlands

**Outlines of Biochemistry**, 3rd Ed. Edited by ROSS AIKEN GORTNER, JR. and WILLIS ALWAY GORTNER. John Wiley & Sons Inc., New York, and Chapman & Hall Ltd., London, 1949. xvi + 1078 pp. Price \$7.50.

According to the claims made on the front cover of the present volume this book contains "a complete discussion of the fundamental organic and physicochemical reactions of plant and animal organisms and the colloidal systems in which they take place" and "covers the fundamental facts which apply to all branches of biochemistry." Also "research workers and advanced students will find the book the most complete reference work in the field of biochemistry." Such statements may have applied to the first edition with which Ross Aiken Gortner, Sr., has done a great service to biochemistry in 1929. They cannot be said to apply to the present third edition. Probably the attainment of such high aims is altogether impossible.

Twenty years ago the extensive treatment of the pure and physical chemistry of colloids, proteins, and carbohydrates was justified since few, if any, suitable textbooks covering these subjects were available in the English language. Today the situation

is totally different. In a certain sense it must therefore be considered as ballast when the chapters discussing these matters take up 736 of the 1016 pages of text. The fundamentals of colloid chemistry as presented on pp. 1—196 can be found in the relevant textbooks. The preparation of ordinary buffer solutions is described in every manual and appears out of place in "Outlines of Biochemistry." Instead of the unimportant  $\text{BaSO}_4$ , from a biochemical point of view, the colloidal or gelatinous  $\text{CaSO}_4$  would have made a better subject of discussion. No mention is made of the large class of hydrotropic substances and the phenomena connected with them except for a passing reference in the chapter on denaturation of proteins. Lengthy discussions on the solubility of  $\text{AgBr}$  and the washing of  $\text{BaSO}_4$  precipitates belong in the field of general and analytical chemistry. Among the mutual precipitation reactions of colloids no mention is made of the biochemically valuable method of deproteinization by means of colloidal  $\text{Fe}(\text{OH})_3$  or kaolin. The tendency of osazones to gel formation, known for many years, is a more instructive phenomenon than the analogous behavior of dibenzoyl cystine. Some shorter sections, on the other hand, are excellent, e.g., that on imbibition pressure referred to on p. 237 with the example of stems of *Alberta opuntia* and the role of water in living organisms, explained on p. 244 with medusa. On p. 488 the phenomenon of alcoholic fermentation of amino acids is indicated implicitly, but the important formation of optically active amyl alcohol in nature is not mentioned. Similarly the conversion of L-glutamic acid to succinic acid via  $\alpha$ -keto-glutaric acid is not described. Regarding the biological method for the resolution of racemic amino acids, it is stated on p. 298 that "this method is rarely of great value in the isolation of the naturally occurring compound." The opposite must be inferred from the methods of asymmetric resolution of ester and acyl derivatives of D-amino acids which have been known for many years and yield both enantiomers. As the best known and most important example of an amino acid with 2 asymmetric C atoms, isoleucine should have been mentioned. As to racemization in the amino acid series, the autoracemization of certain arginine peptides is a remarkable case in point. The polyaspartic acids prepared before 1900 are well defined (cf. p. 301), but no mention is made of the more recently obtained poly-amino acids (Leuchs, Katschalski, Woodward) although their biochemical significance is beyond any doubt. The same is true for the keratins formed by the action of reducing substances on keratin. A criticism of the Bergmann-Niemann theory would be in order. The otherwise extensive description of methods of hydrolysis does not include the fundamental directions of Hlasiwetz and Habermann, dating back to 1873, for the hydrolysis of proteins in the presence of  $\text{SnCl}_2$ . One also misses a discussion of the problem of natural occurrence of D-glutamic acid which could have been inserted on p. 421.

If alkaloid reagents are to be included at all, phosphotungstic acid and phosphomolybdic acid as well as  $\text{KBiI}_4$  should not be lacking, and lead acetate is wrongly designated as a universal reagent.

In contrast to the clearly presented and instructive chapter on carbohydrate metabolism by Paul D. Boyer (pp. 675–711) the other chapters on carbohydrates can not be called satisfactory. The remarks concerning the hydrocyanic acid reaction of sugars and their inability to combine with  $\text{NaHSO}_3$  are incorrect. In the discussion of the osazone reaction no mention is made of the formulation of Fieser, which is probably the best, or of the fact that osazone reactions have now been made quantitative

and the difficulty of poor yields overcome. It is questionable whether a book on biochemistry should include references to the Weerman degradation which does not have even preparative significance. It is equally a matter of opinion whether the formulations of sugar enols and their dissociation, some of which have not been definitely established, should take up many pages of discussion in a biochemistry text.

There is hardly any doubt, however, that certain essential facts should have been considered: No information is given about natural and synthetic optically active fats, naturally occurring optically active fatty acids derived from proteins by bacterial degradation, isomeric inositols known besides the meso form, inososes, amine oxides, recently found aromatic constituents of urine, acetaldehyde, the surprising occurrence of noradrenaline, the biochemically most interesting chloromycetin with organically bound chlorine and a nitro group in a natural compound, *etc.* There is no mention whatsoever of penicillin, streptomycin, and other antibiotics, the presence of fructose in semen and the blood of the foetus, galactogen in beef lung, the natural occurrence of mercaptans, sulfides, disulfides, sulfones, sulfoxides, sulfinic acids and thietines, dehydropeptides and dehydropeptidases and the important specificity of lipases, both natural and under the influence of added substances, contrary to the statement on p. 788 that they do not show any marked specificity. One misses further a discussion of glucuronidase, a description of the various sulfatases, any mention of conjugated sulfuric and glucuronic acids, although these substances have gained renewed interest through researches of recent years on the elimination of sulfa drugs and sex hormones. No statements are made regarding the newer color reactions of the sugars; the claim that the well known color reactions of sugars are due to furfural is out of date. It is not true that the first sulfonic acid in nature was found in 1935 when asterubine was discovered, since taurine was detected 108 years earlier by Gmelin and Tiedemann in 1827. In the list of amino acids generally found in nature,  $\beta$ -aminovaleric acid should be included in view of its relationship with proline and ornithine, the more so since it was the first aminocarboxylic acid found in nature in which the two functional groups are not vicinal to each other. On p. 316 it is stated that both a free-NH<sub>2</sub> and a free-COOH group are required for the violet ninhydrin color reaction; this is definitely incorrect. The possibility that cystine may be the limiting amino acid in wool products (cf. p. 389) had been assumed much earlier than 1932 when Zuntz in 1916 proposed the use of humagsolan for bald-headedness and ovagsolan for sheep feeding. A discussion of the question whether dulcitol is to be considered D- or L- hexitol is just as devoid of any basis as the designation of glycerol or mesoerythritol as D- or L- would be, since these alcohols are derived by reduction of both D- and L- glyceraldehyde or D- and L-erythrose. The same applies to the unsuitable designation D-ribitol and D-xyitol (p. 597). The statements that D-glyceraldehyde and dihydroxy-acetone occur in nature are inexact since only the phosphorylated trioses occur as intermediates. Galacturonic acid (p. 600) is not a constituent of chondroitin sulfuric acid; possibly glucuronic acid has been confused with galactosamine. Designation of the Mg complex in chlorophyll as a salt should be avoided. The paragraphs concerned with photosynthesis are not too satisfactory; they describe the state of knowledge as per 1941 and even that without consideration of Warburg's findings. Nothing is said about intermediary products of photosynthetic carbohydrate formation.

Historical data are not essential, but if they are included they should be correct:

On p. 283 it is stated that the concept of amino acids as inner salts or "zwitter" ions dates back to 1916. Both terms as well as the respective formulations resulted from a number of studies published 1875-1909. The subject was treated under this title in 1913 in Victor Meyer-Jacobson, part Ib, p. 733-734. As for the technical hydrogenation of oils, a patent had been granted long before 1926 to Leprince and Siverke and already incorporated the use of finely divided nickel for this purpose. Cystine had been discovered as a protein constituent in 1899 by Möerner, isoleucine in 1903 by F. Ehrlich. Such well known names as Kithne (p. 278), Bourquelot (p. 613), and F. Buchner (p. 984) should be spelled correctly.

The chapters on lipides and essential oils and on plant pigments and biochemical regulators provide a useful introduction. The shorter sections on biogenesis of flavones, the role of carotenoids, inheritance of color, and hormones in plants contain a good deal of interest. It is regrettable that phytochemistry has not been treated as adequately in the other chapters. Mention of the enzymatic degradation of lignin would have been desirable; this has been elucidated by Fernandez & Regueira, Nord & Brauns and illustrated by the enzymatic formation of vanillin.

In spite of obvious deficiencies the book contains some readable chapters. Its use will depend on individual requirements.

CARL NEUBERG, New York, N. Y.

**Clinical Chemistry in Practical Medicine.** By C. P. STEWART, Reader in Clinical Chemistry, University of Edinburgh, and D. M. DUNLOP, Professor of Therapeutic and Clinical Medicine, University of Edinburgh. The Williams and Wilkins Company, Baltimore. 1949. viii + 324 pp. Price \$5.00.

This book contains a very clear description of the tests used for investigating renal, gastric, pancreatic, and hepatic functions, basal metabolic rate, and metabolism of water, salts, and carbohydrates. As the authors state in their preface, the book does not attempt a complete account of the innumerable tests proposed. However, all the important tests are described and extensively discussed. The technique of those tests which are performed on the patient is described in full detail, whereas the usual analysis of blood, urine, gastric juice and feces is briefly outlined in an appendix. The authors discuss extensively the physiological bases of the clinical tests and their interpretation. Many of their conclusions are based on personal experience and supported by new impressive diagrams. The reviewer does not agree fully with the theoretical treatment on pH and buffers which might lead to the erroneous view that a considerable part of carbonic acid is present as  $H_2CO_3$ . Apart from this and other minor points, however, the book is certainly outstanding by its sound criticism of the different tests. The well-known order for "complete" urine analysis is condemned as a "cloak for ignorance," and blood sugar determinations are considered as unnecessary in most cases of diabetes. These and other similar remarks are certainly justified and necessary with regard to the uncritical viewpoint of many treatises on clinical chemistry. The reviewer recommends this book as an excellent outline, which is particularly valuable for its critical treatment of the whole field of clinical chemistry.

FELIX HAUROWITZ, Bloomington, Indiana

**Plant and Animal Biochemistry.** By EDWIN T. MERTZ and JOHN W. PORTER, Assistant Professors of Agricultural Chemistry, Purdue University, Lafayette, Indiana. Burgess Publishing Co., Minneapolis 15, Minn., 1949. v + 198 pp. Price \$3.25.

As stated in the preface, this loose-leafed textbook has been designed to give the undergraduate the fundamentals of both plant and animal biochemistry. It is expected to provide a broad foundation for students of agriculture, biology, chemistry, home economics or premedicine. The reviewer finds that the authors accomplished this, insofar as is possible in a brief volume.

The book is written in a style that is plain, terse and easy to understand. The one and only serious fault is the absence of a chapter on enzymes.

The reviewer wonders what the evidence is that maltose is synthesized in the starch-forming organs of plants and animals (p. 18); here "synthesized" is used in the strict sense of "building up."

On p. 21 polysaccharides are divided into two groups, according to whether they are digestible or indigestible by plant and animal enzymes. This is a little too strict, since all polysaccharides are digestible, provided the proper enzyme is employed. On p. 39 extracellular enzymes are said to be produced mainly by microorganisms. This is not exactly true. On p. 41 glucose is shown to be converted in muscle metabolism directly to glucose-1-phosphate, whereas it goes first to glucose-6-phosphate, which is then changed to glucose-1-phosphate.

The description of the digestive systems in non-ruminants, ruminants and birds given on pp. 46, 47, and 48 is excellent. On p. 52 blood glucose is said to react with ATP and phosphorylase to give glucose-1-phosphate. This is incorrect. On p. 76 it is stated that glucose is preferentially burned by the liver. This statement means little or nothing to the reviewer.

Tiselius should be mentioned on p. 101 in connection with electrophoresis. On p. 111 it is stated that peptones are nondialyzable. The reviewer has always considered peptones to be dialyzable.

On p. 127 the distinction between an enzyme, a coenzyme and an apoenzyme is not clearly shown. Instead of "Enzyme + Coenzyme" the authors might have written:

$$\underbrace{\text{Apoenzyme} + \text{Coenzyme}}_{\text{Enzyme}}$$

On p. 139 the authors state that vitamins are needed in small amounts in the diet for growth and the maintenance of life, but they do not say why. They might have stated that, in many instances, vitamins are employed as coenzymes, usually after phosphorylation.

On p. 146 the structural formula for cocarboxylase has one phosphate too many.

An excellent discussion of hormones is presented on pp. 165-167.

At the end of the book there is a good subject index.

JAMES B. SUMNER, Ithaca, N. Y.

**Elsevier's Encyclopaedia of Organic Chemistry. Vol. 12 B. Naphthalene. Part I. Hydrocarbons and Halogen Compounds.** Edited by E. JOSEPHY and F. RADT. Elsevier Publishing Company, Inc., New York and Amsterdam, 1946. xxx + 366 pp. Subscription price, \$24.00, Series \$28.00, single \$32.00.

The fourth volume of *Elsevier's Encyclopaedia of Organic Chemistry* to appear consists of 30 introductory pages, 344 pp. of text and 22 pp. of Subject and Formula Indices. It forms a complete compendium of all naphthalene hydrocarbons and their halogen derivatives up to the end of 1944; the literature concerning questions of structure is up-to-date as of 1948. The prospectus promises 6 more parts of Vol. 12B, which are scheduled to appear between now and 1951, and which will deal with nitrogen, hydroxy, oxo, and other naphthalene derivatives. They will complete not only Vol. 12B, but, at the same time, Series III, Carboisocyclic Condensed Compounds.

A perusal of the volume before us impresses the reader not only with the beautiful and clear make-up of the text and the formula panels, but, in particular, with the wisdom of the arrangement which brings, *e.g.*, naphthalenes with one side-chain, with two side-chains, *etc.*, together in individual sections. Each of these sections contains substances of identical skeleton, but different degrees of saturation, side by side; those interested in comparisons of substituted naphthalenes on the same level of saturation will find the dihydro, tetrahydro, *etc.*, compounds exhaustively cross-indexed in the "Summaries" at the beginning of the volume. The complete literature references are chronologically listed at the end of each section; these bibliographies may be easily found by those not yet familiar with the style of the Encyclopaedia, by consulting the tables of contents on p. 1 for Hydrocarbons and p. 239 for Halogeno Compounds. Many other hints for the proper use of the Encyclopaedia are given at the beginning of the volume. There are added as loose leaves a list of Additions and Corrections and several short reprints from *Recueil* on experimental work by the Editorial Staff, dealing with "Discrepancies in the Literature," the facts to be inserted in subsequent parts of Vol. 12B.

The authors of reference works must remain reporters rather than reformers. Thus, they have to record the official designations of compounds, even in cases where the accepted nomenclature could stand improvement; cases in point are ionene (p. 164), which is not an unsaturated ionane, but a cyclization product of the monocyclic ionone; the same holds for irene (p. 195). Although "tetralin" and "decalin" were originally trade names, they have become semi-systematic trivial names and invite the spelling "tetralene" and "decalene." However, such questions of nomenclature must be decided by the proper agencies.

It is in the nature of the naphthalene group that it comprises relatively few natural products. As this chapter of primarily synthetic chemistry will unfold in the following parts of Vol. 12B, the absence of patent references will make itself felt; this will not be as serious as in the case of anthracene, since much of the knowledge gathered in the naphthalene field by the continental industry was never made the subject of patent applications, but remained in the files of the companies, to be frequently rediscovered in academic institutions.

The majority of substituted hydronaphthalenes contain at least one asymmetric carbon atom; thus, a great many compounds, described in the volume, might have been designated "DL." The "radical" nature of di-*p*-biphenyl- $\alpha$ -naphthyl-methyl (p. 237) is neither emphasized in the structural formula nor in the text.

The conspicuous features of arrangement and presentation confirm the impression obtained by the use of the preceding volumes: we have here a system best adapted to the use of the synthetic chemist, based not merely on elementary composition, but on the teachings of structural chemistry. Thus, it will replace in some respects the famous old text of Meyer and Jacobson. It testifies to the experience and foresight of the authors that they were able to achieve this aim by a treatment which is at the same time 100% systematic, and thus assigns a unique place to each and every compound. The system proves not only the most rational in the face of the overwhelming number of compounds which have accumulated in organic chemical literature during the last decades, but, following the principles of modern education, it also meets the requirements of the teacher and of the student.

HARRY SOBOTKA, New York, N. Y.

**Biological Applications of Tracer Elements.** Cold Spring Harbor Symposia on Quantitative Biology, Vol. XIII, The Biological Laboratory, Cold Spring Harbor, L. I., New York, 1948. xi + 222 pp. Price \$7.00.

The monograph presently reviewed is a compilation of the scientific papers delivered at the Cold Spring Harbor Symposium held from June 8 to June 16, 1948. In contrast to other recent conferences concerned with the measurement and production of radioisotopes, the discussion at this meeting emphasized the applications of labeled elements and compounds to biological problems. Since tracers can be applied to a variety of biological problems, it is understandably difficult to achieve any unity in the subject matter under such a general title. The use of tracers is essentially a method of measurement and, in an exaggerated sense, the title bears a resemblance to, let us say, the "Biological Applications of Gravimetric Determinations." The semblance of subject continuity and readability which might have been preserved under these circumstances has been dissipated by the arrangement of the papers alphabetically according to the names of the authors. The reviewer should admit that a belated reading of the preface disclosed that the disarrangement was a calculated one with the expressed objective of improving the book's usefulness as a reference volume. Supposing that one is prepared to discount the serious inconvenience to the large number of readers who read the book for the first time, it is, nevertheless, urged that subject arrangement is preferable for a reference volume, viz., the *Chemical Handbook* and the *Encyclopedias*. *Who's Who* is the only common reference volume that comes to mind employing arrangement by proper names. The reviewer has indulged in some emphasis on this matter since the papers included in the monograph are generally excellent and well worth reading in their entirety. The volume is presented in its usual red cover format. It is amply illustrated by graphs and plates. The reduced graph size only in a single instance (Fig. 7, pg. 169) impairs easy readability. In view of the number of chemical formulae and mathematical derivations, the editor is to be congratulated on a fine job of proofreading. It should be mentioned that the preface is followed by a list of those attending the conference, including some 15 foreign scientists.

The symposium consists of 25 scientific papers giving a reasonably complete picture of present day application of tracers to biological problems. The prospective cover-to-cover reader should certainly begin with the historical sketch of the use of tracers contributed by Hevesy (referred to in the discussion following his paper as "not only one of the fathers of the isotope technique but also the attending gynecologist").

One of the most straightforward applications of radioactive isotopes is the study of genetic changes in plants or seedlings which have been grown in media containing  $P^{32}$ . In this use,  $P^{32}$  is, in fact, used as an internally located radiation source and not as a tracer. Two papers report on this subject. Results which are susceptible of general application are possible only if radiation dosage is measured at the site of the genetic changes recorded. This problem has been solved in one of the two sets of experiments.

Another type of straightforward application of radioisotopes is exemplified in an experiment in which radioactive calcium, strontium, and radium are administered to separate sets of experimental animals. The elimination in the urine and feces is measured and the retention is compared. Experiments of somewhat similar design are reported in which  $C^{14}O_2$  is incorporated in a rat and the rate of elimination is studied as a function of time. A third paper in this group is concerned with the biological half-life of radioactive sodium as followed in 12 human subjects.

A third class of problem attacked by tracer technique is the rate and mechanism of transport of ions and water through the cellular membranes. Suitable experimental design and interpretation of results frequently present difficulties. Six papers appear in this symposium devoted either wholly or in part to the solution of transport problems.

A fourth type of problem for which tracers are of tremendous value is the study of metabolism processes. Eight papers reported in the symposium are partly or wholly concerned with metabolic problems. Experiments are reported investigating the metabolism of purines, glycine and other amino acids, fatty acids, carbohydrates and phosphate.

A fifth general problem for which isotopes may, in some cases, be even uniquely applicable is the synthesis of compounds in the body or in plants. Some 5 papers in the symposium are devoted to studies involving synthesis. The classes of compounds studied are carbohydrates (by photosynthesis), glycogen, lipides, and porphyrins.

In addition to the papers which may be classified in terms of the above general problems, the symposium monograph contains a general literature review of isotopes in pharmacodynamics, which notes that pharmacologists have been slow to make full use of this tracer tool. Several papers report experimental observations but are mainly concerned with the general exposition of ways in which tracers may be usefully applied *viz.*, a paper on the use of  $O^{18}$  and a second paper discussing the unique advantages of conducting experiments using bacteria as the biological material.

Before concluding this review, the reviewer proposes to arbitrarily sample the rich store of experimental data contained in the monograph and present for consideration two general biological problems that are raised and one experimental finding.

In the paper on the use of  $O^{18}$ , the author discusses the Dole effect, *i.e.*, that water prepared from atmospheric oxygen and normal hydrogen had a greater density than fresh water. This finding is of particular interest to biologists, since it is believed that atmospheric oxygen has been largely formed by photosynthetic processes and, therefore, from nutrient water. The question as to whether the plant in the photosynthetic process is able to fractionate oxygen isotopes is presently at issue with results of different experimenters in disagreement.

In the paper on radium metabolism, the authors report the puzzling finding that the retention of radium in rats is greater the greater the dose for a dose range from 0.02 to

0.93  $\mu\text{c/g}$ . Apart from the implications of this finding in respect to the mechanism of radium deposition in bone, it is of interest to health physicists concerned with the occupational hazards of radium ingestion.

The experimental finding singled out for attention is contained in the paper on capillary permeability, where the statement is made that, in the guinea pig, 140% of the plasma water is exchanged each minute with extravascular water and 60% of the plasma sodium and chloride is exchanged each minute. This finding is particularly mentioned since it is a part of a new concept of body processes, emphasizing their essentially dynamic nature. This concept has introduced a new manner of thinking and a new terminology, *i.e.*, the "metabolic pool" conceived as a reservoir of body materials in constant flux. The revolution in the thinking about metabolic processes in the body has been largely brought about by the results of tracer experiments such as are presented in the collection of papers reviewed.

JOHN B. HURSH, Rochester, N. Y.

## Errata

Volume 16, Number 3, p. 339

'*Lactobacillus brevis* (8257) should read, "*Lactobacillus brevis* (8287)."

Volume 25, Number 1, p. 226

"H. N. Horowitz" should read, "N. H. Horowitz."

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